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PRINCIPAL INVESTIGATOR: Matthew R. Yudt, Ph.D.

CONTRACTING ORGANIZATION: University of Rochester
Rochester, New York 14642

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_____ Matthew R. Yudt, Ph.D.

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Matthew R. Yudt, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Rochester
Rochester, New York 14642

E*Mail: yudt@niehs.nih.gov

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13. ABSTRACT *(Maximum 200*

A biochemical analysis of a tyrosine 537 to phenylalanine mutation in the human estrogen receptor (hER) revealed no significant changes in DNA or hormone binding affinities compared to wild type receptor. However, the Y537F hER mutation resulted in altered hormone binding kinetics and a decreased receptor stability, as measured by a loss of hormone and DNA binding over time relative to wild type receptor. Phosphorylation of Y537 is not essential for hER function but Y537 is nevertheless a critical residue intricately involved with the conformation and ability of the hER to activate transcription.

A phosphotyrosine peptide derived from the hER sequence surrounding Y537 and containing part of helix 12 in the ligand binding domain is capable of blocking specific hER-DNA binding *in vitro* . The phosphopeptide inhibition of DNA binding requires the phosphotyrosine and the amino acids carboxy terminal to it. Analysis of hER deletion fragments suggests the ligand binding domain is required for phosphopeptide inhibition. The phosphopeptide does not bind to or compete with the ligand binding site.

The structural information of the ligand binding domain was exploited and a 'dimer-contact' oligopeptide was derived from the helical region of the wild type protein involved in receptor dimerization. This peptide, or I-box peptide, is capable of specifically precipitating the hER from cell extracts. Precipitation activity is correlated with the helical nature of the peptide: a peptide containing a secondary structure-disrupting proline residue has no effect on hER function.

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9/14/99

PI - Signature Date

Table of Contents

INTRODUCTION

The human estrogen receptor (hER) is a member of the steroid/thyroid hormone receptor superfamily of ligand activated transcription factors. The hER functions as a homodimer which binds specific DNA response elements (EREs) in the promoter regions of estrogen responsive genes. The molecular mechanisms by which these receptors regulate transcription is an area of considerable research focused on diverse areas including protein-protein interactions with transcriptional intermediaries. The recently solved three dimensional structure of the hER ligand binding domain (LBD) has lead to a greater understanding and interest in developing drugs targeting the estrogen binding site. The overall research objectives described here are aimed at exploiting known structure-function relations to develop novel approaches at regulating hER function.

The foundation for this proposal stems from previously published reports from Notides laboratory have which proposed a phosphotyrosine mediated dimerization mechanism for the hER (1-4). In this model, tyrosine 537 (Y537) phosphorylation of one monomer interacts with another tyrosine phosphorylated monomer to constitute an hER dimer. Supporting this hypothesis is the important observation that a 12-amino acid phosphotyrosine peptide, derived from the wild type protein sequence surrounding Y537, is capable of blocking receptor dimerization.

<u>The specific aims of this project proposal are</u>,

1. Use molecular and biochemical approaches to identify and isolate the minimal region of the hER sufficient for phosphotyrosyl mediated dimerization.

2. Develop a yeast two-hybrid system to characterize the *in vivo* function of the pY binding domain and dimerization deficient mutants.

3. Determine the recognition specificity of the pY-pY binding domain using phosphotyrosyl peptides and mutagenesis of the domain.

4. Structurally characterize the pY binding domain and peptide interactions using nuclear magnetic resonance (NMR).

BODY

The major conclusions of this research are:

1. A tyrosine 537 to phenylalanine mutation (Y537F) reduces hER protein stability and attenuates receptor-mediated transcriptional response in yeast by approximately 25%.

1.1 . Tyrosine 537 phosphorylation is not essential for the function of the hER dimerization.

1.2. The Y537F hER exhibits altered hormone binding kinetics and an attenuated DNA binding capacity in the presence of estradiol *in vitro*.

The data supporting these conclusions are found in the attached manuscript entitled "The Function of Estrogen Receptor Tyrosine 537 in Hormone Binding, DNA Binding and Transactivation". This manuscript is currently *in press* in the journal Biochemistry. A portion of that data is also part of the yearly report from June 1997-July 1998 (Year 2).

Relation to statement of work (page 9):

These conclusions follow from the **Technical Objectives 1 and 2** as stated in the statement of work (SOW) below. The initial proposal to identify and characterize a phosphotyrosine binding domain and characterize the interactions in a yeast based "two-hybrid" assay were modified to include a thorough biochemical characterization of the role of tyrosine 537 (phosphorylation) in hER hormone binding, DNA binding, and transcriptional activation.

2. A phosphopeptide(Yp11)-hER interaction exists which requires the presence of the LBD. The phosphopeptide Yp11 is derived from the sequence surrounding Y537 and includes a portion of the helix 12 region involved with transcriptional regulation.

2.1. The hER-Yp11 interaction is not a covalent linkage, transcends electrostatic contacts (an unphosphorylated or tyrosine sulfate peptide fails to function similarly), does not

compete with DNA in binding the hER or interfere with the hormone binding site, and does not appear to promote hER aggregation or proteolysis.

2.2. The specific amino acids within the phosphopeptide necessary for inhibition of hER-ERE (and presumably dimerization) are located carboxy terminal to the phosphorylated tyrosine residue and correspond to residues in the amino terminal portion of helix 12.

2.3. Yp-11 peptide can be minimized to the carboxy terminal 7 amino acids and still function as an hER inhibitor. All of the amino acids within the 7-mer are apparently required for maximal inhibition, as site specific alanine substitutions and scrambled sequences fail to function as hER inhibitors.

The data supporting these conclusions were published in my thesis dissertation entitled "The Role of Tyrosine 537 in Estrogen Receptor Function and the Development of Rationally Designed Peptide Antiestrogens". The third manuscript from this thesis which specifically regards the phosphopeptide-hER inhibitors remains 'in preparation'. A portion of that data is also part of the yearly report from June 1996-July 1997 (Year 1).

Relation to statement of work (page 9):

These conclusions follow from the **Technical Objective 3** as stated in the statement of work (SOW) below. The peptide interaction region was found to include the ligand binding domain (LBD) of the hER and the proposed mutagenesis was limited to alterations of the peptide sequence until further information regarding specific contacts with the hER LBD can be determined and the appropriate mutagenesis designed.

3. A second peptide sequence (IB-wt) derived from the hER H10/11 region, i.e. the dimer interface, also functions as an antiestrogen *in vitro*, by specifically precipitating the hER from protein mixtures. Interestingly, this peptide contains an LXXLL motif, important in mediating hER protein-protein interactions with coactivators.

3.1. The ability of the IB-wt peptide to block hER function by precipitating the protein is dependent on the helical nature of the peptide as determined from circular dichroism studies where a single Ile to Pro mutation in the helical region of the I-box peptide significantly reduces the helical content and abolishes the precipitation activity.

3.2. The precipitation effect of the IB-wt is specific for the hER as the peptide does not act similarly on the related progesterone or androgen receptors (PR, AR) or other general proteins in solution.

3.3. A homologous peptide derived from the nuclear receptor RXRα dimer interface, and a helical LXXLL-containing peptide from the coactivator TIF2, have no detectable *in vitro* effect on hER function or solubility. Our data suggest that rationally designed molecules capable of affecting steroid receptor quaternary structures may be potential avenues for the development of specific inhibitors of this class of proteins

The data supporting these conclusions are found in the attached manuscript entitled "Preventing Estrogen Receptor Action with Dimer-Interface Peptides". This manuscript was submitted to the Journal of Biological Chemistry in April 1999 and is currently undergoing revision to include more *in vivo* data supporting these conclusions (ref 6). A portion of that data is also part of the yearly report from June 1997-July 1998 (Year 2).

Relation to statement of work (page 9):

These conclusions follow from the **Technical Objective 4** as stated in the statement of work (SOW) below. The initial proposal to study the phosphopeptide-hER interaction by nuclear magnetic resonance (NMR) was not carried out because: *a*) the region

of interaction was found to be the ligand binding domain (LBD) *b*) the hER LBD is too large to be studied at high resolution by NMR *c*) the structure of the LBD has been determined by X-ray crystallography. As a very suitable alternative, the interesting discovery regarding the helix 10/11 dimer interface (I-box) peptide was studied in further detail and compared with the phosphopeptide discussed above.

The initial statement of work submitted with this proposal is shown below:

Statement of Work

Technical Objective 1.

By the end of month 2 have identified the phosphotyrosine binding (PTB) domain of the hER and expressed in bacterial system

Technical Objective 2.

Task 1. Months 3-6: Construction of yeast strains and development of vectors for two-hybrid system.

Task 2. Months 7,8: Characterization PTB domain utilizing two-hybrid system.

Technical Objective 3.

Task 1. Months 11,12: Develop in-vitro assay to quantify peptide affinities/specificities.

Task 2. Months 13-15: Determine specificity and affinity of PTB domain for peptide ligands

Task 3. Months 16-20: Mutational analysis of PTB domain.

Technical Objective 4.

Task 1. Months 21,22: Overexpress, solubilize and purify hER PTB domain

Task 2. Months 23-30: Determine high resolution, atomic scale image of hER PTB by NMR.

Task 3. Months 31-36: NMR analysis of peptide/PTB interaction.

Month 37 Write Ph.D thesis

Month 38 Defend Ph.D thesis

REFERENCES

1. Arnold, S.F. and A.C. Notides. *An antiestrogen: a phosphotyrosyl peptide that blocks dimerization of the human estrogen receptor.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(16): p. 7475-9.

2. Arnold, S.F., D.P. Vorojeikina, and A.C. Notides, *Phosphorylation of tyrosine 537 on the human estrogen receptor is required for binding to an estrogen response element.* Journal of Biological Chemistry, 1995. **270**(50): p. 30205-12.

3. Arnold, S.F., *et al.*, *Phosphorylation of the human estrogen receptor on tyrosine 537 in vivo and by src family tyrosine kinases in vitro.* Molecular Endocrinology, 1995. **9**(1): p. 24-33.

4. Arnold, S.F., *et al.*, *Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation.* Molecular Endocrinology, 1997. **11**(1): p. 48-53.

5. Yudt M.R., Vorojeikina, D., Zhong, L., Skafar, D.F., Sasson, S., Gasiewicz, T.G., and Notides, A.C. *The Function of Estrogen Receptor Tyrosine 537 in Hormone Binding, DNA Binding and Transactivation* Biochemistry, *in press.*

6. Yudt, M.R., and S.Koide. Preventing Estrogen Receptor Action with Dimer-Interface Peptides. *submitted, undergoing revision.*

7. Yudt, M.R. **The Role of Tyrosine 537 in Estrogen Receptor Function and the Development of Rationally Designed Peptide Antiestrogens.** University of Rochester Thesis Dissertation, successfully defended December 1998.

APPENDIX

1. Key Research Accomplishments

- The role of tyrosine 537 in the function of the human estrogen receptor (hER) was determined, settling a controversial topic regarding the function of this amino acid in hER signaling.
- A tyrosine 537 to phenylalanine mutation in the hER attenuated the transcriptional activation of the hER, significantly altered the hormone binding kinetics of the hER, and altered the DNA binding capacity of the hER in the presence of estradiol.
- The specificity of hER-inhibitory tyrosine phosphorylated peptides was characterized.
- A dimer-interface peptide was designed which specifically blocks hER action by precipitating the protein from complex mixtures.
- A specific phosphotyrosine binding domain was not found within the estrogen receptor, however binding of the hER to phosphotyrosyl peptides requires the ligand binding domain (LBD).

- It is intended that three (3) manuscripts will be published as first author resulting from this predoctoral fellowship.
- The first manuscript is in press in Biochemistry and is considered a 'solid addition to the field' regarding the role of tyrosine 537 in function of the human estrogen receptor.
- A second manuscript is under revision for the Journal of Biological Chemistry and concerns the ability of a dimer-contact oligo peptide to block hER action.
- A third manuscript regarding the specificity and mechanism of phosphotyrosine containing peptides to block hER function is in preparation and undergoing a collaborative effort with scientists at the University of Rochester who will continue this project into the future.
- The work from this fellowship was published as a thesis dissertation and was successfully defended in December 1998 at the University of Rochester, Rochester, NY.

2. Reportable Outcomes:

a. Manuscript 1. Yudt M.R., Vorojeikina, D., Zhong, L., Skafar, D.F., Sasson, S., Gasiewicz, T.G., and Notides, A.C. *The Function of Estrogen Receptor Tyrosine 537 in Hormone Binding, DNA Binding and Transactivation* Biochemistry, *in press.*

b. Manuscript 2. Yudt, M.R., and S.Koide. Preventing Estrogen Receptor Action with Dimer-Interface Peptides. *submitted, undergoing revision.*

c. Manuscript 3. Yudt, M.R., Koide, S.K. and Notides, A.C. Specificity and mechanism of phosphotyrsoyl peptide inhibition of hER function *in vitro*. In preparation

d. Thesis Dissertation. Yudt, M.R. **The Role of Tyrosine 537 in Estrogen Receptor Function and the Development of Rationally Designed Peptide Antiestrogens.** University of Rochester Thesis Dissertation, successfully defended December 1998

e. Abstracts presented:

Yudt, M.R., D. Vorojeikina, A.C. Notides, and S. Koide. The Role of Tyrosine 537 in Estrogen Receptor Function and the Development of Rationally Designed Peptide Antiestrogens. AACR Special Symposium on Steroid Hormone Receptors, Jan. 1999, Palm Springs, CA.

Yudt, M.R., and S. Koide. A Rationally Designed Peptide Antiestrogen. University of Rochester Cancer Center Symposium. Rochester, NY. October, 1998

Yudt, M.R., Castano, E.C., and A.C. Notides. New Antiestrogens: Phosphopeptides Which Inhibit Estrogen Receptor Function by Disrupting Dimerization. 'Era of Hope' Conference, Washington D.C. Oct. 1998.

Yudt, M.R., Castano, E.C., and A.C. Notides. Disruption of Estrogen Receptor Dimerization and DNA-Binding by a 12-mer Phosphotyrosyl Peptide Requires the C- but not N- Terminal Amino Acids. Keystone Symposia, Lake Tahoe, NV; 1996

f. Oral presentations

New Antiestrogens: Phosphopeptides Which Inhibit Estrogen Receptor Function by Disrupting Dimerization; Oct 1997 - "Era of Hope" Breast Cancer Research Conference; Washington D.C.

The Regulation of Estrogen Receptor Function by Phosphorylation; Dec. 1996 - University of Pittsburgh, Reproductive Physiology (presented in place of Dr. A.C. Notides)

g. Degrees

Ph.D. degree in Biophysics awarded May 15, 1999. University of Rochester, Rochester NY.

j. Employment obtained based on experience gained during fellowship: A post-doctoral fellowship at the NIH/NIEHS in Research Triangle Park, NC, in the laboratory of Dr. J. Cidlowski was obtained based on the successful completion of my Ph.D. dissertation and the training obtained under funding of this fellowship.

k. Awards.

As part of the exceptional training under this fellowship, the author was the recipient of William F. Neuman Award in Biophysics at the University of Rochester, 1998.

Supplemental material attached:

1. Manuscript 1: Yudt M.R., Vorojeikina, D., Zhong, L., Skafar, D.F., Sasson, S., Gasiewicz, T.G., and Notides, A.C. *The Function of Estrogen Receptor Tyrosine 537 in Hormone Binding, DNA Binding and Transactivation* Biochemistry, *in press.*

2. Manuscript 2: Yudt, M.R., and S.Koide. Preventing Estrogen Receptor Action with Dimer-Interface Peptides. *submitted, undergoing revision.*

3. Thesis Dissertation Exerts - included are: title page, curriculum vitae, abstract, table of contents, and list of figures and tables.

4. Abstract 1. **Yudt, M.R.**, D. Vorojeikina, A.C. Notides, and S. Koide. The Role of Tyrosine 537 in Estrogen Receptor Function and the Development of Rationally Designed Peptide Antiestrogens. AACR Special Symposium on Steroid Hormone Receptors, Jan. 1999, Palm Springs, CA.

5. Abstract 2. **Yudt, M.R.**, and S. Koide. A Rationally Designed Peptide Antiestrogen. University of Rochester Cancer Center Symposium. Rochester, NY. October, 1998

6. Abstract 3. **Yudt, M.R.**, Castano, E.C., and A.C. Notides. New Antiestrogens: Phosphopeptides Which Inhibit Estrogen Receptor Function by Disrupting Dimerization. 'Era of Hope' Conference, Wahington D.C. Oct. 1998.

7. Abstract 4. **Yudt, M.R.**, Castano, E.C., and A.C. Notides. Disruption of Estrogen Receptor Dimerization and DNA-Binding by a 12-mer Phosphotyrosyl Peptide Requires the C- but not N-Terminal Amino Acids. Keystone Symposia, Lake Tahoe, NV; 1996

The Function of Estrogen Receptor Tyrosine 537 in Hormone Binding, DNA Binding and Transactivation[†]

Matthew R. Yudt[‡], Daria Vorojeikina, Li Zhong[§], Debra F. Skafar[§], Shlomo Sasson[||], Thomas A. Gasiewicz*, and Angelo C. Notides[⊥]

Departments of Biochemistry and Biophysics, and Environmental Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642; Department of Physiology and the Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI 48201; Dept. of Pharmacology, Hebrew University School of Medicine, PO Box 12272, Jerusalem, 91120

Running title: hER tyrosine 537 and helix 12 mutations

[†] This work was supported by NIH grant HD06707 and Center Grant ES01247. MRY was supported by a U.S. Army MRMC breast cancer research pre-doctoral fellowship (DAMD 17-96-1-6219). DFS was supported by grants from the NSF (IBN-940736), the Karmanos Cancer Institute and the National Cell Culture Center.

* To whom correspondence should be addressed. Phone: (716) 275-7723, Fax (716) 256-2591. Email: 'gasiewt@envmed.rochester.edu'.

[‡] Department of Biochemistry and Biophysics. Present address: NIEHS, P.O. Box 12233, MD E202; Research Triangle Park, NC, 27709.

[§] Wayne State University

[||] Hebrew University

[⊥] This manuscript is dedicated to the memory of the late Professor Angelo C. Notides. MRY and DV contributed equally to this work.

FOOTNOTES

[1] Abbreviations: hER, human estrogen receptor alpha; LBD, ligand binding domain; AF-2; activation function 2; ERE, estrogen response element; WT, wild type; Y537F, tyrosine 537 to phenylalanine; TBE, Tris-borate-EDTA; TDEG, Tris-DTT-EDTA-glycine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; H12, helix 12; SEM, standard error of the mean.

[2] There are two forms of the estrogen receptor, alpha and beta. The estrogen receptor alpha was used for the studies described here, and hER denotes the alpha form, unless otherwise stated.

ABSTRACT

The human estrogen receptor (hER) is a ligand activated transcription factor which, like many other members of the nuclear receptor protein family, exhibits a dimerization-dependent transcriptional activation. Several previous reports have provided evidence for the phosphorylation of the hER at tyrosine 537 (Y537). However, the exact function of a putative phosphorylation at this site remains controversial. Using a yeast transactivation assay, and *in-vitro* biochemical approaches, we show that phosphorylation of tyrosine 537 is not required for the hER to bind hormone, or to activate transcription. An hER tyrosine 537 to phenylalanine (Y537F) mutant retains 70-75% of the transactivation potential of wt hER in a yeast reporter system. Furthermore, the mutated receptor exhibits wild type hormone and DNA binding affinities. However, this mutation results in a decrease in receptor stability as measured by a decrease in hormone binding over time. The most striking difference between the wt and Y537F hER is in the estradiol binding kinetics. Whereas the off-rate for estradiol displays a two-state binding mechanism, the Y537F mutant hER exhibits a monophasic estradiol off-rate. Based on these data and other reports describing the structure and activity of Y537 mutations, as well as knowledge of the three dimensional structure of the hER ligand binding domain, we propose an alternate model where Y537F mutation favors an 'open' pocket conformation, affecting the estrogen binding kinetics and stability of the hormone-bound, transcriptionally active 'closed' pocket conformation. Although its phosphorylation is not essential for function of the hER, Y537 is nevertheless a critical residue intricately involved with the conformational changes of the hER and its ability to activate transcription.

The human estrogen receptor (hER)[2] is an essential component of a variety of signal transduction pathways, culminating in the transcription of estrogen-responsive genes (*1*). Multiple signaling factors are involved in the regulation of hER-mediated transcription, including the type of ligand bound (*2*), the interaction of the receptor with other protein co-regulators (*3*), and the phosphorylation status of the receptor (*4, 5*).

The hER, like all other members of the nuclear hormone receptor superfamily, is composed of multiple structural and functional domains (*6*). The ligand binding domain (LBD) not only binds its target ligands, but contains a large dimerization interface, and a hormone dependent transactivation function, termed AF-2 (*7*). The transcriptionally active hER is a homodimer. The AF-2 can be defined as the surface generated by the conformation of the carboxy-terminal alpha helix (helix 12, hER amino acids 538-545) formed upon ligand binding. Crystal structure analysis has shown that different classes of ligands confer distinct and opposite orientations of this helix, and hence generate different binding surfaces for the transcription intermediary co-activator proteins (*8, 9*).

Peptide mapping and western blotting studies using antiphosphotyrosine antibodies provide suggestive evidence for Y537 phosphorylation (*10, 11*). Several hypotheses have been developed for the function of Y537 phosphorylation. In calf uterine cytosol and MCF-7 breast cancer cells, Y537 phosphorylation reportedly plays a role in the hormone binding activation of hER (*12*). Other studies have proposed additional roles for Y537 phosphorylation in receptor homodimerization, a prerequisite for DNA binding and transactivation (*10, 13*).

Recently, the physiological importance of hER Y537 was suggested by the detection of a naturally occurring tyrosine to asparagine mutation (Y537N) at this site in a metastatic breast carcinoma (*14*). When transfected into mammalian cell cultures, the resulting Y537N mutant hER exhibited a potent estradiol-independent activation. This result was corroborated by two independent reports characterizing constitutively active

mouse and human estrogen receptors mutated at Y537 (*15, 16*). This ligand-independent activation phenomenon has been directly correlated with the binding of co-activators to the unliganded hER (*17, 18*). However, in those studies, a tyrosine to phenylalanine mutant (Y537F) did exhibit hormone dependent transcriptional activation, similar to the wild type (wt) protein (*15, 16*).

To resolve these apparently contradictory reports, we have continued our study of hER Y537. We report here that the Y537F mutation does not significantly alter hER affinity for either the estrogen response element (ERE) or estrogenic ligands. The Y537F hER exhibits a modest 25-30% decrease in hER-mediated transcription in a yeast expression-reporter assay. The recombinant Y537F hER appears less stable than the wt in the presence and in the absence of estradiol. These observations are partially explained by the demonstration of altered estradiol binding kinetics between the wt and Y537F hER. In summary, we demonstrate that Y537 and its phosphorylation are not essential for hER dimerization and function, but nevertheless contribute to critical conformational changes and protein stability.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]Estradiol (50 Ci/mmol) and [^{32}P]-γ-ATP (3000 Ci/mmol) were purchased from Dupont-NEN (Boston, MA). The ICI 182,780 was kindly provided by Dr. A. E. Wakeling (Zeneca Pharmaceuticals, Mereside, UK). Tamoxifen citrate was obtained from Stuart Pharmaceuticals (Wilmington, DE). Glass beads, 0.5 mm in diameter, for breaking yeast cell walls, were purchased from Biospec (Bartesville, OK). Bio-Rad's Muta-Gene in vitro mutagenesis kit was used for site-directed mutagenesis and the dideoxy sequenase kit from USB corp. was used for sequencing. Anti-hER polyclonal antibody 6, directed

against amino acids 259-278 (*19*) was affinity purified for use in western blotting, and reacted equivalently to all hER mutants described in the text. Secondary antibodies were purchased from Santa Cruz Biotech.

Site-directed mutagenesis

Oligo-directed mutagenesis of the hER was performed by the method of Kunkel (*20*). The hER cDNA (HEGO) was cloned into M13mp19 to produce single stranded DNA. Oligonucleotides used for mutagenesis were: Oligo #1-Y537F-5'-CAG-CAG-CTC-GAA-GAG-GGG-CAC-CAC-3'; Oligo #2-L540Q-5'-CAT-CTC-CAG-CTG-CAG-CTC-ATA-GAG-GGG-5'. Y537F mutated DNA was used for the second round of mutagenesis together with oligo #2 to synthesize the double mutant Y537F/L540Q (dm). All mutations were verified by DNA sequencing. Mutated hER cDNA was cloned into the EcoR1 site of both the yeast expression vector pSCW231(*21*) and the baculovirus transfer vector pVL1393 (Invitrogen, San Diego CA). The orientation of cloned inserts was verified by restriction enzyme digests with BglII and SmaI.

Preparation of recombinant hERs

Spodptera frugipedra (Sf9) cells were infected with baculovirus containing wt or the mutant hER cDNA, grown for 4 days at 27 C, and lysed by repeated freeze/thaw cycles in a hypotonic buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 0.5 mM PefaBlock (Boehringer Manheim), and 80 μM leupeptin, pH 7.4). KCl was added to a final concentration of 500 mM and extracts were incubated on ice for 30 minutes before centrifugation at 28,000 X g. Supernatants were either used as soluble cell extracts, or the hER partially purified by 40% ammonium sulfate precipitation. Protein extracts from *Saccharomyces cerevisiae* were prepared by the glass bead method according to Kaiser et al. (*22*).

Hormone binding assays

Ammonium sulfate precipitated hERs were used with final hER concentrations of 2 or 10 nM in a TDEEK buffer (40 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM EDTA, 1 mM

EGTA, 0.2 mM PefaBlock, 0.5 mM leupeptin, 15% glycerol, and 150 mM KCl). Ovalbumin was added to achieve a final protein concentration of 5 mg/ml. For saturation binding experiments the receptor preparation was incubated with various concentrations of [^{3}H]estradiol (0.3 - 100 nM). Non-specific binding was measured by a parallel incubation with a 200-fold molar excess of unlabeled estradiol. Following incubation for 16 hr. on ice, an aliquot of each mixture was removed to determine the total [^{3}H]estradiol concentration. The unbound hormone was removed by incubation with dextran-coated charcoal solution (0.03%/0.3% final concentrations). Following liquid scintillation counting, the specific binding was obtained by subtracting non-specific from total binding. The K_d was determined by Scatchard analysis.

Electrophoretic mobility shift assays

Whole cell extracts or ammonium sulfate fractions of *Sf9* produced hER were prepared as described above. The gel mobility experiments were performed by incubation of equivalent amounts (approx. 6 nM) of wt or mutant receptor extracts in binding buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 0.2 mM Na_3VO_4) with protease inhibitors (1 mM leupeptin and 0.5 mM PefaBlock). Final concentrations of salt and protein were maintained at 100 mM and 0.2 mg/ml, respectively. A double stranded 27-base pair probe was end labeled with ^{32}P-γ-ATP, gel purified, and specific activity determined using thin layer chromatography and liquid scintillation counting (1-10 fmoles = 20,000 cpm). One µg of poly(dI-dC) (Pharmacia Biotech.) was used to block non-specific DNA binding. Following a one hour incubation on ice, the samples were electrophoresed on a 5% non-denaturing polyacrylamide gel for 2.5 hours, 175 V, 4 C, in a 0.5X TBE running buffer. The gels were dried and exposed to PhosphorImager plates. Band intensities were quantified using ImageQuant software (Molecular Dynamics) and saturation plots created and analyzed using Sigma Plot. For Scatchard analysis, bands were excised and analyzed by Cherenkov counting.

Yeast strains, growth conditions and transfections

The *Saccharomyces cerevisiae* yeast strain 939 was used was used for all experiments (*23*). Yeast cells were grown in minimal yeast medium (0.67% [w/v] yeast nitrogen base without amino acids and 2% [w/v] glucose) supplemented with the required amino acids. Yeast were transformed by the lithium acetate procedure (*24*). The cells were then plated on minimal yeast medium supplemented with leucine (100 mg/ml). In these transformations, plasmid DNA consisted of a 1:2 mixture of the yeast expression vector pSCW231-hER or appropriate mutant hER and the reporter plasmid YRPE2 (*25*). This plasmid contains two copies of a consensus estrogen responsive element upstream of the cyc promoter linked to a *lac Z* reporter gene. Successfully transformed yeast cells were able to restore auxotrophy and grow on synthetic glucose minimal plates without uracil and tryptophan (SD/-ura-trp).

Quantitative β galactosidase assays

Yeast cells from a single colony were grown overnight at 30 C to an OD_{600} of 1.0 - 1.5. Following dilution to an OD_{600} of 0.1, 2 ml aliquots were transferred to 18 mm diameter glass tubes where 20 µl of the appropriate ligand solution was added. Incubation was continued in an orbital shaker at 250 rpm for 16 hours. The ligand treated cells were collected by centrifugation and β-galactosidase activity was assayed using whole, permeabilized cells. The values were expressed in Miller units as previously described (*21*).

Western blots

Western blots were performed as described previously (*19*), using a polyclonal antibody raised in our laboratory for hER detection. Quantification of band intensities was done by densitometry using a range of protein concentrations and multiple film expqsures. For antiphosphotyrosine blotting, the manufacturer's recommended protocol was followed. Several antiphosphotyrsoine antibodies were used, including the PY20 presented here, and PY66 antibodies from Santa Cruz Biotechnology (Santa Cruz, CA), as well the 4G10 antibody from Upstate Biotechnology (Lake Placid, NY).

Stability of the hormone-free receptor

The wt and mutant Y537F hER expressed as histidine-tagged fusions in Sf9 insect cells were used for these experiments in addition to the non-histidine-tagged receptor. Cell pellets were lysed in TDEG buffer plus 20 mg/ml ovalbumin and 0.4 mM PMSF, 0.1 mM leupeptin, 1.5 μM aprotinin and 2 μM E64 in a Dounce tissue homogenizer and centrifuged at 220,000 X g for 30 minutes. Soluble extracts were then incubated in the absence of hormone at 25 C for up to 4 hours with aliquots being removed at the indicated times. Hormone binding activity was measured after a one hour incubation at 0 C with labeled estradiol as described above.

Dissociation of [^{3}H]estradiol from the wt and Y537F mutant hER

Extracts from Sf9 insect cells expressing either the wt or Y537F mutant hER were prepared and incubated with 20 nM [^{3}H]estradiol for 60 min at 25 C. The unbound steroid was removed by incubation with 0.5 volumes of dextran-coated charcoal suspension in TDE buffer for 10 min on ice. The charcoal was pelleted by centrifugation (5 min, 1000 X g). The supernatant was removed and 10 μM unlabeled estradiol was added and the incubation was continued at 25 C. At the times indicated, 0.2 ml aliquots were removed and incubated with 0.1 ml of the dextran-coated charcoal suspension for 10 min. on ice. The charcoal was pelleted with centrifugation and 0.1 ml of the supernatant was removed

for measurement of bound [^{3}H]estradiol using liquid scintillation counting. The inactivation of the receptor was measured in parallel incubations in which the first dextran-coated charcoal treatment was eliminated.

The data were analyzed using GraphPad PRISM, and were fit to either a two-phase or a one-phase exponential decay as indicated. The values of the fast dissociation component were corrected by subtraction of the contribution of the slow phase component. All values were plotted semilogarithmically, with the Y-axis being the percentage of bound [^{3}H]estradiol.

RESULTS

Estrogen receptor mutations in the AF-2-core region of the hormone binding domain.

The mutation of tyrosine 537 to phenylalanine was initially created to investigate the putative role of phosphorylation on this residue in receptor dimerization and activation (*13*). A second mutation, L540Q, was also prepared, which has been documented to disrupt transcriptional co-activator interactions with the hER and displays a dominant negative phenotype (*26, 27*). A third hER mutant, Y537F/L540Q, containing the mutations at both residues was also prepared. By studying the Y537F mutation alone and in the context of a second mutation not only close by, but with an established phenotype, the role of this residue in receptor function can be further dissected. Figure 1 shows the location of these residues in relation to the known secondary structure associated with the region.

Transcriptional activation of wild type and mutant estrogen receptors in yeast.

A yeast expression-reporter system was constructed to investigate the transcriptional activation properties of the wt and mutant hERs (*21*). The hERs expressed

in yeast were transcriptionally active and hormone responsive (Fig. 2). The relative transcriptional activity of wt and mutant hERs were measured after treatment with 10 nM estradiol (Fig. 2a). The Y537F, L540Q and Y537F/L540Q double mutant (dm), showed an approximately 25%, 50%, and 75% respective decrease in transactivation relative to wt. Yeast cells containing an expression vector lacking the hER cDNA or those containing a cDNA inserted in the opposite orientation (inverted) showed no estradiol response.

A western blot with a purified anti-hER polyclonal antibody indicated a consistent and equivalent level of the hER protein in all strains (Fig. 2b). Although random deviations observed in yeast expressed wt and mutant hERs can be quantified by densitometry of western blots (within 10-20%), the observed differences in transactivation remained consistent. No hER protein was detected in yeast cells lacking the hER cDNA expression vector or in the cDNA inverted strain. Thus, differences in transcriptional activity were not due to differences in the levels of expressed protein.

Maximal activation of transcription occurred near 10 nM estradiol for each mutant (Fig. 2c). Higher concentrations of ligand had little effect, indicating that the mutant proteins, even when stimulated maximally by estradiol, do not attain the same levels of activation as wt.

Transcriptional activation of hERs by selected agonists and antagonists.

We compared the ligand specificity and effectiveness of various hER agonists and antagonists in intact yeast cells. As shown in figure 3, the activation of lac z by both wt and mutants was strictly estrogen specific. No induction of β-galactosidase was detected in the presence of other steroids; progesterone, dexamethasone, or testosterone (data not shown). Estriol was able to stimulate reporter expression at 10-fold higher concentrations than were required for maximal induction with estradiol. The antiestrogens ICI 182,780 and tamoxifen citrate had no effect on wt or mutant hER transactivation when added alone but were able to antagonize the estradiol induced transactivation. The responses to these

compounds in yeast is in good agreement with published mammalian cell culture and *in vivo* data (*28, 29*). These data suggest that none of the mutations significantly alters the ligand binding properties of the hER.

Estradiol binding analysis of wt and mutant hERs

The hER mutants studied here were also produced in baculovirus-infected *Sf9* cells in order to obtain sufficient quantities of receptor for binding analyses. Receptors produced in *Sf9* and yeast cells exhibit similar characteristics to the native hER (*19*). The wt and mutant baculovirus hERs exhibited similar [^{3}H]estradiol binding affinities (Kd = 0.8-2.0 nM) at both low and high hER concentrations (Fig. 4). We compared the cooperativity of the mutants with wt hER to address the question of either mutation affecting the necessary site-site interactions attributed to the cooperative nature of estradiol binding (*30*). At concentrations between 10-12 nM, all hERs displayed maximum cooperativity with a Hill coefficient of 1.4 (± 0.02-0.05). At intermediate concentrations (2-4 nM hER) the affinity and observed partial cooperativity (Hill coefficient 1.1-1.3) were also similar. These experiments were repeated 2-3 times with different receptor preparations under identical conditions, with no significant deviations. Low concentrations of hERs (<1 nM) showed no cooperative estradiol binding and did not vary significantly in relative affinities (figure 4 insets).

The Y537F mutation alters the capacity but not the affinity of the hER for an estrogen response element (ERE)

The *Sf9* produced hERs were analyzed by electrophoretic mobility shift assays to determine if the altered transcriptional responses of the mutant hERs were a result of variations in their DNA binding properties. Cell extracts were first standardized for hER content by western blotting and densitometry to determine relative concentrations, to within 10% error, of total hER among the receptor preparations (data not shown). Using

equivalent amounts of total hER, the apparent ERE affinity was estimated from saturation curves with ^{32}P-labeled ERE in both the presence and absence of estradiol. Saturation and Scatchard plots for the wt and mutants were generated by maintaining equivalent receptor and protein concentrations and increasing the amount of ERE added to the reaction (Fig. 5). The wt and mutant hERs exhibited similar affinities for the ERE in either the absence or presence of estradiol. It should be emphasized that the amount of hER used in these experiments was calibrated on the basis of the total number of receptors as quantified by western blotting, which will not necessarily represent equivalent hormone binding sites. The exact relationship between binding sites and receptor stability is unclear, but it is likely that a mutation-induced loss of protein stability would decrease the number of hormone binding sites, and result in an apparent decrease in the concentration of active (hormone-binding) receptor.

A distinct difference in DNA binding capacity was observed between the wt and Y537F mutant proteins upon treatment with estradiol. After pre-incubation of wt hER with estradiol the maximum binding to the ERE increased more than two-fold over non estradiol treated samples, while the apparent K_d remained essentially unchanged. An identical estradiol incubation with an equivalent amount of Y537F receptor had only a small effect on ERE binding (Fig. 6). This result suggests the stabilizing effect of estradiol on the hER is absent in Y537F mutant.

Y537 stabilizes the hormone-binding activity of the hER

The difference in protein stability between the wt and Y537F was tested directly in two ways. First, cell extracts containing the wt and Y537F mutant hER were prepared in the absence and in the presence of carrier protein (ovalbumin, 20 mg/ml). The presence or absence of carrier protein had little effect on the binding of the wt hER to [^{3}H]estradiol (Fig. 7a). However, in the absence of the carrier protein, the Y537F mutant hER displayed little [^{3}H]estradiol binding activity; the activity was increased approximately 10-fold by the

addition of carrier (Fig. 7a). Western blotting revealed no differences in the recovery of hER protein, or sensitivity to proteolysis (not shown).

Second, cell extracts expressing either wt or Y537F receptor and containing carrier protein were incubated for up to 4h at 25 C in the absence of hormone, followed by measurement of the remaining hormone binding activity. The wt hER retained its hormone binding activity essentially unchanged for up to 4 h, whereas the Y537F hER lost 30% of its binding activity during that time (Fig. 7b). This effect was most striking in extracts prepared using low ionic strength buffer. In contrast, when cells were prepared in the presence of 300 mM KCl, the hormone binding activity of both receptors increased approximately 3-fold during the first hour of incubation (Fig. 7c). The activity of wt receptor remained stable for up to 4h. The hormone binding activity of the Y537F hER remained near maximal for up to 2h, but a small (approx. 15%) decrease from maximal levels was observed at 4h (figure 7C). Western blotting revealed no detectable differences in immunoreactivity between aliquots taken at the beginning or the end of the incubation (not shown).

Dissociation of [^{3}H]estradiol from the wt and Y537F mutant hER

The dissociation of [^{3}H]estradiol from the wt hER showed a biphasic, or two-component dissocation curve (Fig. 8). The half life of the first, fast component was 13.0 ± 0.6 min, while the half life of the second, slow component was 118 ± 3 min. By contrast, the dissocation of [^{3}H]estradiol from the Y537F mutant hER appeared linear, with a half life 65 ± 9.8 min (Fig. 8). Moreover, when the data for dissocation from the Y537F were fit to equations for two-phase dissocation and one-phase dissociation, the one-phase dissocation gave a better fit (not shown). No effect of receptor concentration on the dissocation kinetics of either the wt or the mutant was observed under these conditions.

DISCUSSION

Tyrosine 537 of the hER is located in a flexible loop region at the base of the amphipathic alpha helix (H12) of the ligand binding domain which undergoes extreme conformational changes in response to hormone binding (*8, 9*). We have studied the function of Y537 by biochemical analysis of specific mutations, and provide a comparison with studies of other relevant mutations within this region (Table 1). We find that a tyrosine to phenylalanine mutation reduces hER stability, alters the hormone binding kinetics, and attenuates receptor-mediated transcriptional response in yeast by approximately 25%. Furthermore, in combination with the dominant negative L540Q hER mutant, the effect on transcription is additive. These data support a hypothesis in which hER Y537 is an important component of the intricate protein structure/function mechanism of hER activation.

Yeast transactivation assays and hER mutants

Yeast-based transcription assays are common approaches to study mammalian hormone receptors (*31*). Yeast expressed hER has been shown to exhibit similar DNA and hormone binding affinities to the mammalian expressed receptor (*32-34*). We have established a transactivation assay for the hER in *S. cerevisae* which is completely hormone dependent and responds to steroids comparably with other reported yeast and mammalian expression systems (*21, 35*). Yeast expressing the receptor exhibit no measurable basal transcriptional activation in the absence of estrogen treatment. Another distinction of the yeast system is that unlike transient transfection assays, a relatively low and consistent amount of hER protein is expressed, estimated around 5-10 nM (A. Notides, unpublished data).

The yeast expressing Y537F hER exhibited a small but measurable decrease in maximum transactivation which was further diminished in combination with the L540Q AF-2 mutant. In our system the mutant and wt receptors were expressed at equivalent

levels (within 10%) independent of added estradiol, and all responded to agonists and antagonists in a manner consistent with mammalian cell cultures or in vivo systems (*36*). Although several yeast studies show a lack of estradiol antagonsim using tamoxifen and ICI 182,780, and in fact report of a partial agonist response with these compounds, we and others have observed estradiol antagonism with these compounds in yeast (*21, 52* and references therein). Several explanations have been proposed to account for these differences, including yeast strain and promoter specificity, different uptake ratios and metabolism of antiestrogen compounds in yeast, and the presence or absence of specific factors associated with transcriptional activation of the hER.

Despite the known tyrosine kinase and phosphatase activity in *S.cerevisiae* (*37, 38*) we have not determined whether in fact the Y537 site of the hER expressed in yeast was phosphorylated. On the other hand, the difference between wt and Y537F obtained with the yeast transcription assay (figure 2a) were quantitatively similar to those obtained in mammalian cell transcription assays performed by us and others (D. Vorojeikina, unpublished data, and ref. *15, 16*). This suggests that the role of Y537 phosphorylation in transcriptional activation is probably insignificant.

Tyrosine 537 Phosphorylation

Tyrosine phosphorylation of the hER was first reported fourteen years ago where it was found that ^{32}P-labeling of the rat uterine estrogen receptors occurred on tyrosine residues (*39*). This receptor also reacted with anti-phosphotyrosine antibodies (*40*). It was shown by deletion and mutational analysis that tyrosine 537 was required for maximal hormone binding activity of *in vitro* synthesized ER (*41*). These authors proposed that tyrosine 537 phosphorylation was required for estradiol activation of the hER.

An alternative role for Y537 phosphorylation was proposed in which tyrosine phosphorylation not only regulates hormone binding activity (*42*), but is also required for the DNA binding and dimerization of the hER (*13*). In this model, Y537 phosphorylation

is essential for the interaction of one monomer with another tyrosine phosphorylated monomer to constitute an active hER dimer. The data presented here, which were verified with multiple preparations of sequence-verified recombinant hER mutants, as well as similar data presented by others (*15, 16*), do not support this hypothesis. Several explanations for the differences from earlier observations exist. The observed decrease in stability of the Y537F hER described here may have been interpreted as an effect of phosphorylation state. Additionally, differences in expression levels during the original preparation of the Y537F mutant could have resulted in an apparent loss of activity when compared to the wt. Since we do observe a decrease in receptor stability with the Y537F, small changes in sample handling and preparation could magnify differences with the wt hER.

Several attempts were made in our laboratories to distinguish the Y537F mutation from wt by western blotting with anti-phosphotyrosine antibodies. Although the phosphotyrosine antibodies do react with the hER (figure 9), no significant differences were observed between wt or Y537F receptors expressed in Sf9 insect cells, suggesting either a lack of tyrosine 537 phosphorylation in the recombinant hER and/or the presence of alternative tyrosine phosphorylated sites. Interestingly, the possibility of alternative tyrosine phosphorylation sites on the hER has recently been suggested (*43*). To date, the only direct evidence for Y537 phosphorylation reported was a result of radiolabel sequencing of the hER tryptic peptides from Sf9 and MCF-7 cells (*10*). However, the amount of phosphorylation on Y537 was only 5-6% of the total hER phosphorylation. Thus, the extent and the functional role of tyrosine 537 phosphorylation therefore remains unclear.

The decreased stability of the Y537F hER can be explained by several lines of reasoning which do not involve phosphorylation. As seen in figure 10, the hydroxyl group of Y537 forms a hydrogen bond with asparagine 348, within helix 3 on the estradiol bound hER (*8*). The potentially stabilizing effect of this interaction in the hormone-bound hER is

obviously absent in the phenylalanine mutant. Furthermore, phenylalanine itself is known to have a helix de-stabilization effect when studied in relation to other hydrophobic amino acids (*44*).

The wt hER, expressed in Sf9 insect cells, exhibits biphasic dissocation kinetics, as has previously been reported for the calf uterine hER (*45*). The values of the half-lives of the two phases are somewhat different for the two receptors, 5.8 and 173 minutes for the calf ER vs 14.9 and 118 for the recombinant hER. We do not know whether this variance is due to differences in the solution conditions between experiments, or reflect actual differences between the bovine and human receptors. The biphasic dissocation kinetics for the wt hER and positive cooperative equilibrium binding reported here for both wt and Y537F mutant are consistent with the ERs being in the dimeric state.

In contrast with the wt hER, the Y537F mutant exhibited monophasic dissocation kinetics, with a half-life of approximately 70 min. These results were surprising, since the equilibrium studies demonstrated that the wt and Y537F mutant hER bound [^{3}H] estradiol with the same affinity. The results suggest that mutation of Y537 affected the association rate, as well as the dissocation rate, of the interaction between estradiol and the ER. This observation is consistent with the conclusions of Carlson et al. (*46*) who studied deletion mutants of the hER and the Y537S mutant of the hER. However, in their report, neither cooperative binding of estradiol to the receptor, nor biphasic dissocation kinetics of estradiol from the receptors were observed. Overall, the data are consistent with the ideas that loss of the predicted hydrogen bond between Y537 and N348 dramatically alters the kinetics of the receptor-hormone interaction. The ability of the Y537 to form a hydrogen bond is also important in the stability of the hormone-free receptor.

Analysis of Y537 and H12 Mutations

Several constitutively active Y537 mutant hERs have been identified in which serine, alanine, glutamic acid and asparagine replace the tyrosine (*14-16*). The degree of

constitutive activity has been correlated to the formation of a stabilized interface for receptor co-activators or transcription factors (*16*). These receptors apparently gain hormone independent function by folding into a 'closed' position where H12 covers the ligand binding pocket (*46*). Our data indicate that the phenylalanine substitution has a small, but opposite, destabilizing effect. We propose a model in which the tyrosine to phenylalanine change favors a more 'open', or a 'loose' conformation in both the presence and absence of hormone (figure 11). The change in hydrogen bonding and the increased hydrophobicity of the phenylalanine could account for the difference between phenylalanine and tyrosine at this position by preventing a stable conformation of the hER. Similarly, the increased size of phenlylalanine, relative to serine and alanine, could explain the lack of Y537F constitutive activity as observed for the Y537S and Y537A hER. This model assumes a two state mechanism for hormone binding for both wt and mutant hERs. Alternatively, the Y537F hER could adopt a distinct conformation in the hormone-free state. While the Y537 mutants are able to bind co-activators (*16*), the L540Q and other helix 12 AF-2 AD mutations, such as the double mutants L539A/L540A and M543A/L544A, do not (*47-49*). Mutations of other neighboring amino acids have been found to affect hER function as well. Constitutive activity has been shown for an L536P hER mutant (*50*), and another mutation, D538V, displays a reduced transcriptional response to estradiol in yeast, similar to the L540Q hER (*51*). These mutations are summarized in table 1 and suggest a dual role for the helix 12 region in providing both a stable and a specific surface for co activators, since these mutations can apparently affect either function (figure 11).

The analyses presented here are intended to clarify the role of tyrosine 537 in hER function. Although the possible phosphorylation of Y537 and the dimerization of the hER are apparently not directly related, mutations at this site do have significant consequences on receptor function. These effects, as well as those seen in mutations nearby, can be partially explained with an understanding of the structure of the LBD and the role of helix 12 in activation.

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REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and et al. (1995) *Cell 83*, 835-9.

2. Parker, M. G., Fawell, S. E., Danielian, P., and Arbuckle, N. (1991) *Biochem. Soc. Trans. 19*, 897-9.

3. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) *Mol. Endocrinol. 10*, 1167-77.

4. Le Goff, P., Montano, M. M., Schodin, D. J., and Katzenellenbogen, B. S. (1994) *J. Biol. Chem. 269*, 4458-66.

5. Denton, R. R., Koszewski, N. J., and Notides, A. C. (1992) *J. Biol. Chem. 267*, 7263-8.

6. Parker, M. G. (1995) *Vitamins & Hormones 51*, 267-87.

7. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) *Cell 59*, 477-87.

8. Brozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature 389*, 753-8.

9. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell 95*, 927-937.

10. Arnold, S. F., Obourn, J. D., Jaffe, H., and Notides, A. C. (1995) *Mol. Endocrinol. 9*, 24-33.

11. Migliaccio, A., Rotondi, A., and Auricchio, F. (1986) *EMBO J. 5*, 2867-2872.

12. Castoria, G., Migliaccio, A., Green, S., DiDomenico, M., Chambon, P., and Auricchio, F. (1993) *Biochemistry 32*, 1740-1750.

13. Arnold, S. F., Vorojeikina, D. P., and Notides, A. C. (1995) *J. Biol. Chem. 270*, 30205-12.

14. Zhang, Q. X., Borg, A., Wolf, D. M., Oesterreich, S., and Fuqua, S. A. (1997) *Cancer Res. 57*, 1244-9.

15. Weis, K. E., Ekena, K., Thomas, J. A., Lazennec, G., and Katzenellenbogen, B. S. (1996) *Mol. Endocrinol. 10*, 1388-98.

16. White, R., Sjoberg, M., Kalkhoven, E., and Parker, M. G. (1997) *EMBO J. 16*, 1427-35.

17. Lazennec, G., Ediger, T. R., Petz, L. N., Nardulli, A. M., and Katzenellenbogen, B. S. (1997) *Mol. Endocrinol. 11*, 1375-86.

18. Kalkhoven E, Valentine Je, Heery Dm, and Parker Mg. (1998) *EMBO J. 17*, 232-43.

19. Obourn, J. D., Koszewski, N. J., and Notides, A. C. (1993) *Biochemistry 32*, 6229-36.

20. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA 82*, 488-492.

21. Chen, C. W., Hurd, C., Vorojeikina, D. P., Arnold, S. F., and Notides, A. C. (1997) *Biochemical Pharmacology 53*, 1161-72.

22. Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory Press, Plainview, NY.

23. Jones, E. W. (1991) *J. Biol. Chem. 266*, 7963-7966.

24. Chen, D. C., Yang, B. C., and Kuo, T. T. (1992) *Current Genetics 21*, 83-84.

25. Pham, T. A., Hwung, Y. P., Santiso-Mere, D., McDonnell, D. P., and O'Malley, B. W. (1992) *Mol. Endocrinol. 6*, 1043-50.

26. Ince, B. A., Zhuang, Y., Wrenn, C. K., Shapiro, D. J., and Katzenellenbogen, B. S. (1993) *J. Biol. Chem. 268*, 14026-32.

27. Ince, B. A., Schodin, D. J., Shapiro, D. J., and Katzenellenbogen, B. S. (1995) *Endocrinology 136*, 3194-9.

28. Sasson, S., and Notides, A. C. (1983) *J. Biol. Chem. 258*, 8113-7.

29. Wakeling, A. E., and Bowler, J. (1988) *Journal of Steroid Biochemistry 31*, 645-653.

30. Notides, A. C., Lerner, N., and Hamilton, D. E. (1981) *Proc. Natl. Acad. Sci. USA 78*, 4926-30.

31. White, J. H., Metzger, D., and Chambon, P. (1988) *Cold Spring Harbor Symposia on Quantitative Biology 53 Pt 2*, 819-828.

32. Metzger, D., White, J. H., and Chambon, P. (1988) *Nature 334*, 31-36.

33. McDonnell, D. P., Nawaz, Z., Densmore, C., Weigel, N. L., Pham, T. A., Clark, J. H., and O'Malley, B. W. (1991) *J. Steroid Bioch. Mol. Biol. 39*, 291-7.

34. Wooge, C. H., Nilsson, G. M., Heierson, A., McDonnell, D. P., and Katzenellenbogen, B. S. (1992) *Mol. Endocrinol. 6*, 861-9.

35. Lyttle, C. R., Damian-Matsumura, P., Juul, H., and Butt, T. R. (1992) *J. Steroid Bioch. Mol. Biol. 42*, 677-685.

36. Tsai, M.-J., and O'Malley, B. W. (1994) *Annual Review of Biochemistry 63*, 451-486.

37. Wilson, L. K., Benton, B. M., Zhou, S., Thorner, J., and Martin, G. S. (1995) *J. Biol. Chem. 270*, 25185-25193.

38. Schieven, G., J., T., and G.S., M. (1986) *Science 231*, 390-393.

39. Migliaccio, A., Rotondi, A., and Auricchio, F. (1984) *Proc. Natl. Acad. Sci. USA 81*, 5921-5925.

40. Auricchio, F., Migliaccio, A., Castoria, G., Rotondi, A., and Di Domenico, M. (1987) *Methods in Enzymology 139*, 731-744.

41. Migliaccio, A., Di Domenico, M., Green, S., de Falco, A., Kajtaniak, E. L., Blasi, F., Chambon, P., and Auricchio, F. (1989) *Mol. Endocrinol. 3*, 1061-1069.

42. Arnold, S. F., Melamed, M., Vorojeikina, D. P., Notides, A. C., and Sasson, S. (1997) *Mol. Endocrinol. 11*, 48-53.

43. Patrone, C., Gianazza, E., Santagati, S., Agrati, P., and Maggi, A. (1998) *Mol. Endocrinol. 12*, 835-841.

44. Padmanabhan, S., Marqusee, S., Ridgeway, T., Laue, T. M., and Baldwin, R. L. (1990) *Nature 344*, 268-270.

45. Weichman, B. M., and Notides, A. C. (1977) *J. Biol. Chem. 252*, 8856-62.

46. Carlson, K. E., Choi, I., Gee, A., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (1997) *Biochemistry 36*, 14897-905.

47. Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992) *EMBO J. 11*, 1025-33.

48. Cavailles, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) *EMBO J. 14*, 3741-51.

49. Mahfoudi, A., Roulet, E., Dauvois, S., Parker, M. G., and Wahli, W. (1995) *Proc. Natl. Acad. Sci. USA 92*, 4206-10.

50. Eng, F. C., Lee, H. S., Ferrara, J., Willson, T. M., and White, J. H. (1997) *Mol. Cell. Biol. 17*, 4644-53.

51. Wrenn, C. K., and Katzenellenbogen, B. S. (1993) *J. Biol. Chem. 268*, 24089-98.

52. Tsai-Pflugfelder, M., Gasser, S. M., and Walhi, S. (1998) *Mol. Endocrinol. 12*, 1525-41.

Figure Legends

Fig. 1. Sequence Surrounding Helix 12 of the hER Ligand Binding Domain.

The boundaries of the structural and functional domains of the hER are illustrated in relation to the helix 12/AF-2 core region in the ligand binding domain. Amino acids mutated in this study are shown in tall bold letters (Y537 and L540). The location of helices 11 and 12 is also indicated by the underlined sequences (*8*).

Fig. 2. Transcriptional Activation of WT and Mutant hER in Yeast

A, The yeast strains containing either the wild type or indicated mutant hER and the ERE containing reporter were grown at 30 C for 18 hr in the presence or absence of 10 nM estradiol. Transcriptional activity of hER was measured by the β-galactosidase assay and are shown as percent of maximal response, measured in Miller units. The data shown are the means ± SEM for 8-20 individual samples from 3-5 different experiments. B, Western blot of yeast expressed hERs. Yeast cells, prepared as above, were lysed in SDS sample buffer, boiled for 5 min., and subjected to western blot analysis with anti-hER antibody. A representative result from 3 experiments is shown. The wt(inv) contains yeast extract in which the yeast expression vector has an inverted orientation of the hER cDNA sequence, resulting in no observable protein expression. C, The ability of wild type and mutant hERs to activate transcription over a range of estradiol concentrations was measured as described in A. Each curve is the mean ± SEM of three separate experiments, carried out in triplicate.

Fig 3. Steroid Specificity of the Mutant and WT hERs.

The yeast cells expressing the wt and mutant hERs along with the reporter plasmid YRPE2 were grown in medium containing the indicated ligand at 30 C for 16 h. E2, 17-β-estradiol; E3, estriol. The ability of the antiestrogens tamoxifen citrate (TAM) and ICI 182, 780 (ICI) to block estradiol-mediated transactivation of the hERs was measured at the concentration of E2 corresponding to an approximately half maximal response; 5 nM for wt and Y537F or 50 nM (*) for L540Q and dm hERs.

Fig 4. Saturation Analyses of the [^{3}H] estradiol binding of the mutant and wt hERs.

Aliquots of extracts containing 0.8-12 nM of receptors were incubated for 16 hr on ice with increasing concentrations of [^{3}H]estradiol. Specific ligand binding was determined by subtraction of non-specific binding in the presence of a 200-fold excess of radio inert estradiol from the total binding observed. The data were analyzed by the methods of Scatchard and Hill. The relative affinities were estimated from fitted saturation plots and did not vary between wt and mutants more than 50% for any given measurement. The Hill coefficients (n_H) are indicated for each receptor with the SEM for 3 independent measurements. A representative Scatchard plot is shown for each mutant at both high and low (insert) concentrations.

Fig. 5. DNA Binding Analysis of WT and Mutant hERs

Relative expression levels of wt and mutants were calibrated by titrating cell extracts over a range of hER concentrations on a western blot. Equivalent amounts of the indicated hERs were then subjected to electrophoretic mobility shifts assays (EMSA). To obtain affinity measurements, constant amounts of cell extract were incubated with increasing concentrations of ^{32}P-labeled ERE probe. Following electrophoresis, hER-ERE and free ERE bands were excised and analyzed by Cherenkov counting. Affinity constants were derived as described by the method of Scatchard. A representative analysis is shown with the calculated affinity constants for that experiment. The mean K_d values obtained from three different experiments ranged from 1-5 nM and were not significantly different.

Fig. 6. The DNA Binding Capacity of the hER is Compromised in Y537F.

A, Equivalent amounts of wt and Y537F hER cell extracts were incubated overnight on ice in both the absence and presence of 10 nM estradiol before incubation with increasing concentrations of ^{32}P-labeled ERE probe. The overnight incubation of wt hER in the absence of hormone did not decrease ERE binding as compared with the same receptor used immediately in a gel shift (data not shown). B, The data presented in A were plotted as a function of ERE concentration. The ordinate is relative units as measured on the PhosphorImager from an equal exposure time for each gel. The relative affinities of wt and Y537F hER do not vary more than 2-fold from experiment to experiment. A representative graph from three separate experiments is shown, with the smooth line indicating the data fit to a hyperbolic equation.

Fig. 7. Stability of the wt and Y537F mutant ERs.

A, Cell extracts were prepared with and without 20 mg/ml ovalbumin carrier protein. The extracts were incubated with 20 nM [^{3}H]estradiol for 1.5 h in an ice-water bath. A parallel incubation containing 20 nM [^{3}H]estradiol and a 200-fold molar excess of unlabeled estradiol was carried out to determine specific binding. Free and bound steroids were separated by dextran-coated charcoal assay, and were measured using liquid scintillation counting. These data are typical of four independent experiments. B, Cell extracts were prepared from Sf9 cells expressing wt or the Y537F hER, in a low ionic strength TDEG buffer containing 20 mg/ml ovalbumin. The extracts were incubated without hormone at 25 C for up to 4 h, and the remaining hormone binding activity measured as described in Methods. The data are expressed relative to the initial concentration of specifically bound [^{3}H]estradiol. The initial concentrations of bound [^{3}H]estradiol were 3.7 and 3.8 nM (wt); 6.7 and 7.3 nM (Y537F). The data are the average of 2 independent experiments. C, Cell extracts prepared as in B, with the addition of 0.3 M KCl. The initial concentrations of bound [^{3}H]estradiol were 1.6 and 3.2 nM (wt); 1.1 and 2.6 nM (Y537F). The data are the average of 2 independent experiments.

Fig. 8. Dissociation of [^{3}H]estradiol from the wt and Y537F mutant hER.

Extracts from *Sf9* insect cells expressing either wt (■) and Y537F (□) hER were prepared and the dissociation of [^{3}H]estradiol from the receptors was measured as described. Receptor concentrations were between 1.05 nM and 2.67 nM for wt and 0.86 nM and 5.1 nM for the Y537F hER. The values shown are the mean and SEM of four independent experiments. The lines represent the best-fit to two-phase exponential decay (wt) or one-phase exponential decay (Y537F). Inactivation of the wt (▲) and Y537F (Δ) hERs was measured in parallel incubations and was less than 5%.

Fig. 9. Reactivity of hER to antiphosphotyrosine antibodies.

To analyze the wt and Y537F hER for reactivity to antiphosphotyrosine antibodies increasing amounts of total cell extracts containing approximately equivalent amounts of both wt and Y537F hER were run on SDS-PAGE and transferred to PVDF membranes. The blots were first probed with the PY20 antiphosphotyrosine antibody from Santa Cruz Biotech (Anti-PY) according to the manufacturer's protocol. The membrane was then stripped and reprobed with the Anti-hER antibody (Ab 6). Similar results were obtained when the order of probing was reversed. Other antiphosphotyrosine antibodies tested also reacted with the hER (see Methods). The raised triangle corresponds to increasing total protein in each lane corresponding to approximately 2.5, 10 and 25 micrograms of total protein. The hER band at 66 kDa is indicated to the right along with the positions of the molecular weight markers (MW, kDa).

Fig. 10. Potential hydrogen bond between Y537 and N348.

Residues Y537 and N348 are shown in magenta; estradiol is shown in yellow. The potential hydrogen bond is indicated by a dotted green line and is highlighted by an arrow. Generated using the coordinates of the hER ligand-binding domain deposited in the PDB (1ere, *8*) using Molecualr Images software.

Fig. 11. A Model Illustrating the Effects of Various Helix 12-Region Mutations on a Hypothetical Pathway of hER Activation.

The activation of the dimeric wild type hER proceeds through a hormone binding event which results in a conformational change of the helix 12 region and the conversion from an open to a closed hormone binding pocket. The indicated mutations of Y537 and the neighboring helix 12 amino acids are proposed to affect either the formation of the closed complex, (left half reaction), or the interaction with coactivators (right half reaction). The constitutively active Y537S, Y537A and L536P are shown in a closed pocket conformation, independent of hormone binding. The Y537F mutation is shown to affect the open/closed conformational equilibrium or form a third distinct conformation. The mutations shown are discussed in the text. In this model, specific amino acids can easily be understood as having a role in either or both activation steps.

FIGURE 1

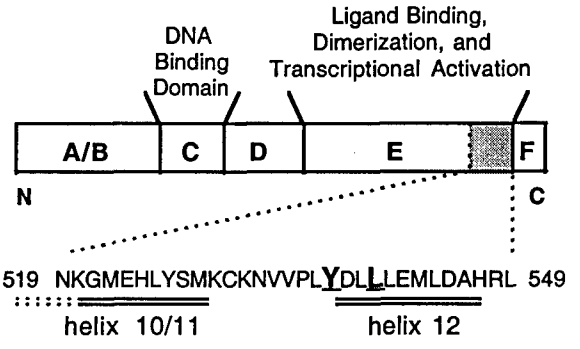
DNA
Binding
Domain
Ligand Binding,
Dimerization, and
Transcriptional Activation
A/B
C
D
E
F
N
C
519 NKGMEHLYSMKCKNVVPLYDLLLEMLDAHRL 549
helix 10/11
helix 12

FIGURE 2

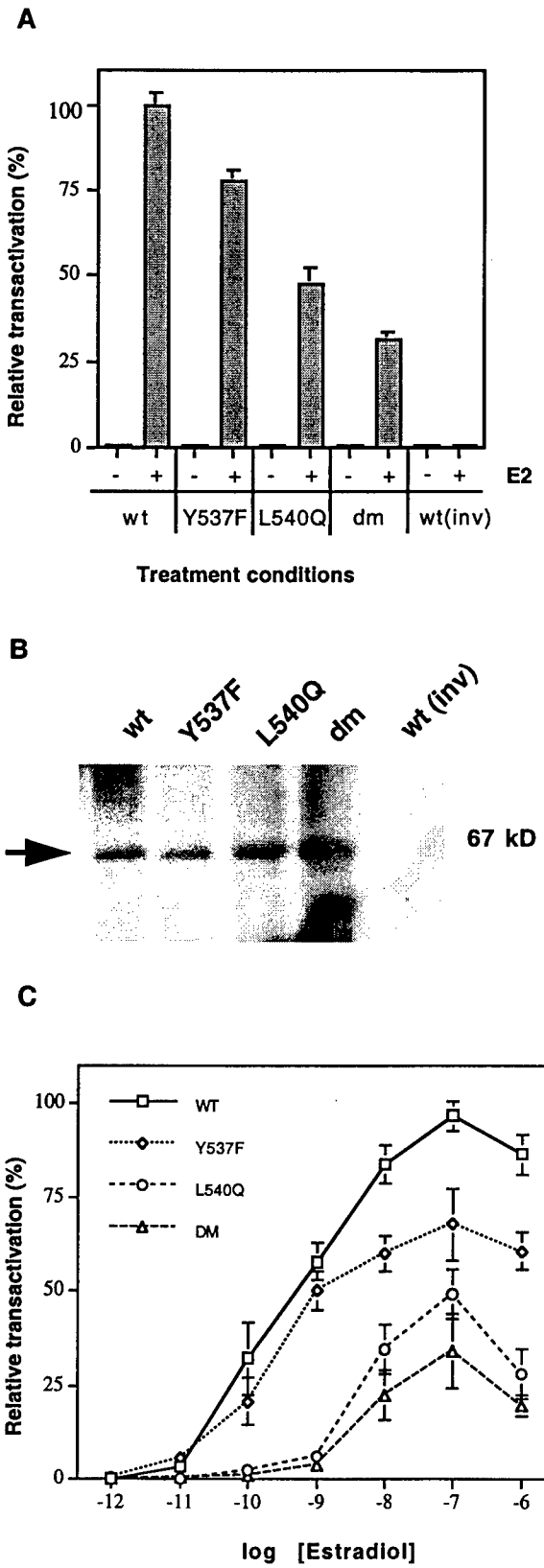
A
Relative transactivation (%)
100
75
50
25
0
- + - + - + - + - +
E2
wt
Y537F
L540Q
dm
wt(inv)
Treatment conditions
B
wt
Y537F
L540Q
dm
wt (inv)
67 kD
C
Relative transactivation (%)
100
75
50
25
0
WT
Y537F
L540Q
DM
-12
-11
-10
-9
-8
-7
-6
log [Estradiol]

FIGURE 3

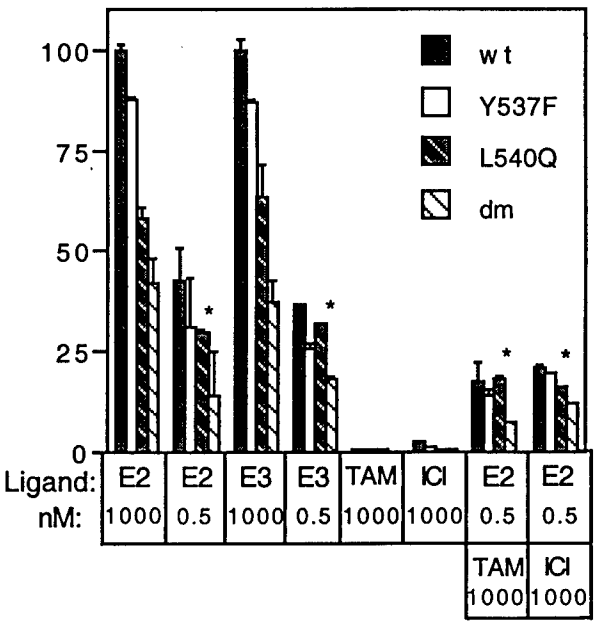
100
75
50
25
0
wt
Y537F
L540Q
dm
Ligand:
nM:
E2 1000
E2 0.5
E3 1000
E3 0.5
TAM 1000
ICI 1000
E2 0.5
TAM 1000
E2 0.5
ICI 1000

FIGURE 4

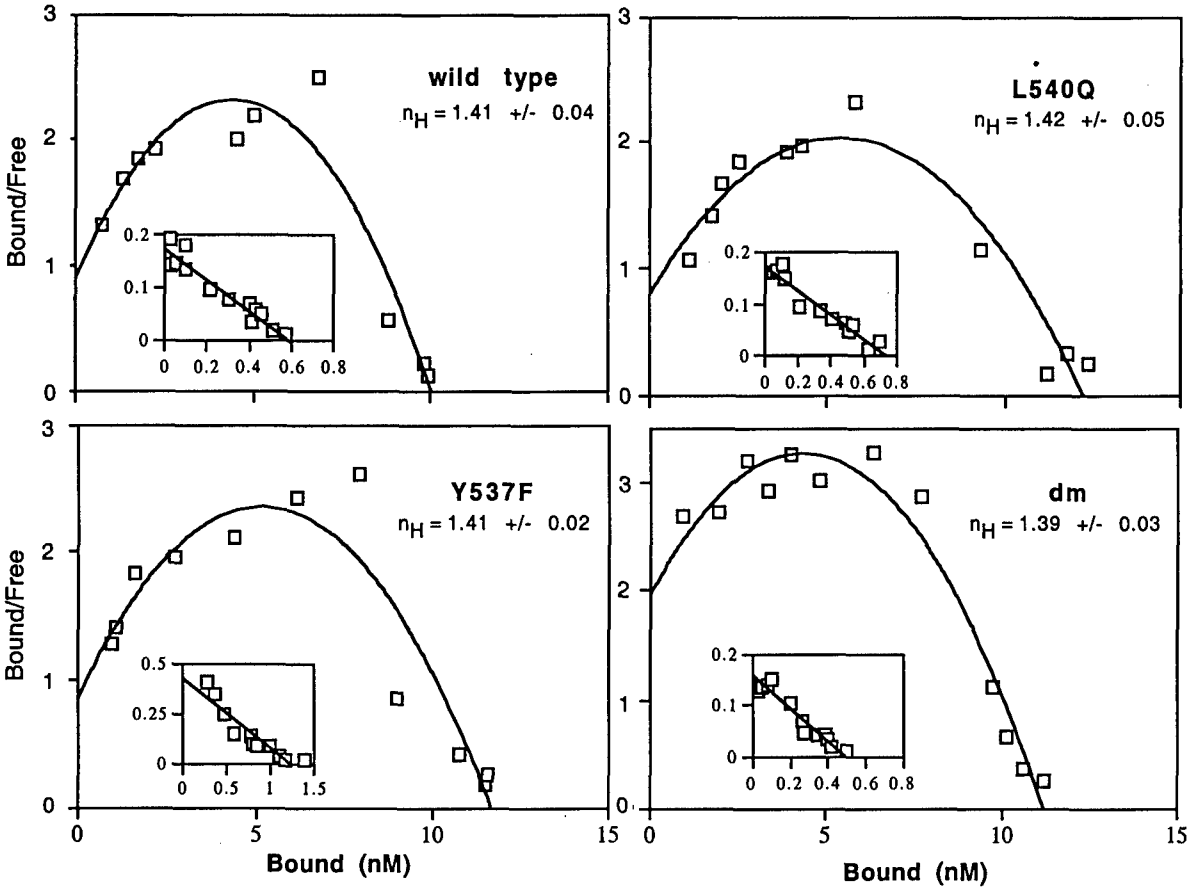
wild type
n_H = 1.41 +/- 0.04
L540Q
n_H = 1.42 +/- 0.05
Y537F
n_H = 1.41 +/- 0.02
dm
n_H = 1.39 +/- 0.03
Bound/Free
Bound (nM)

FIGURE 5

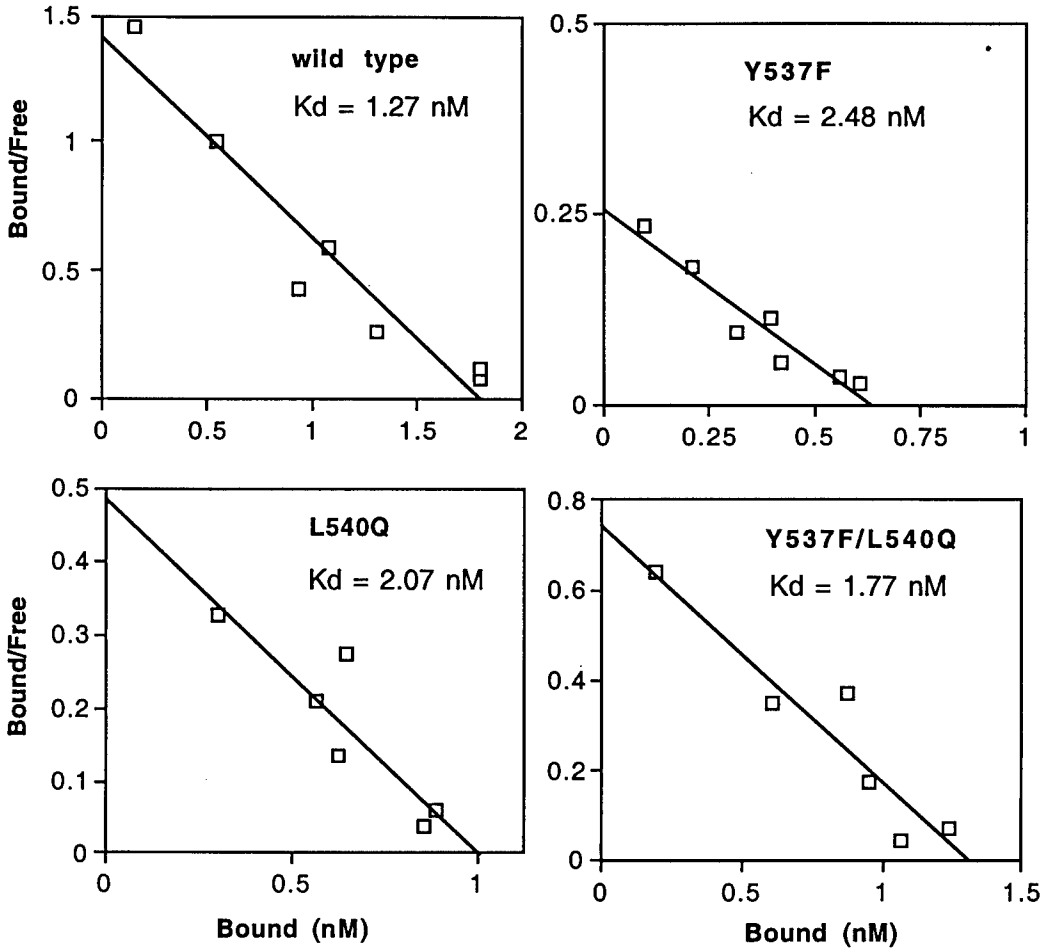
wild type
Kd = 1.27 nM
Y537F
Kd = 2.48 nM
L540Q
Kd = 2.07 nM
Y537F/L540Q
Kd = 1.77 nM
Bound/Free
Bound (nM)

FIGURE 6

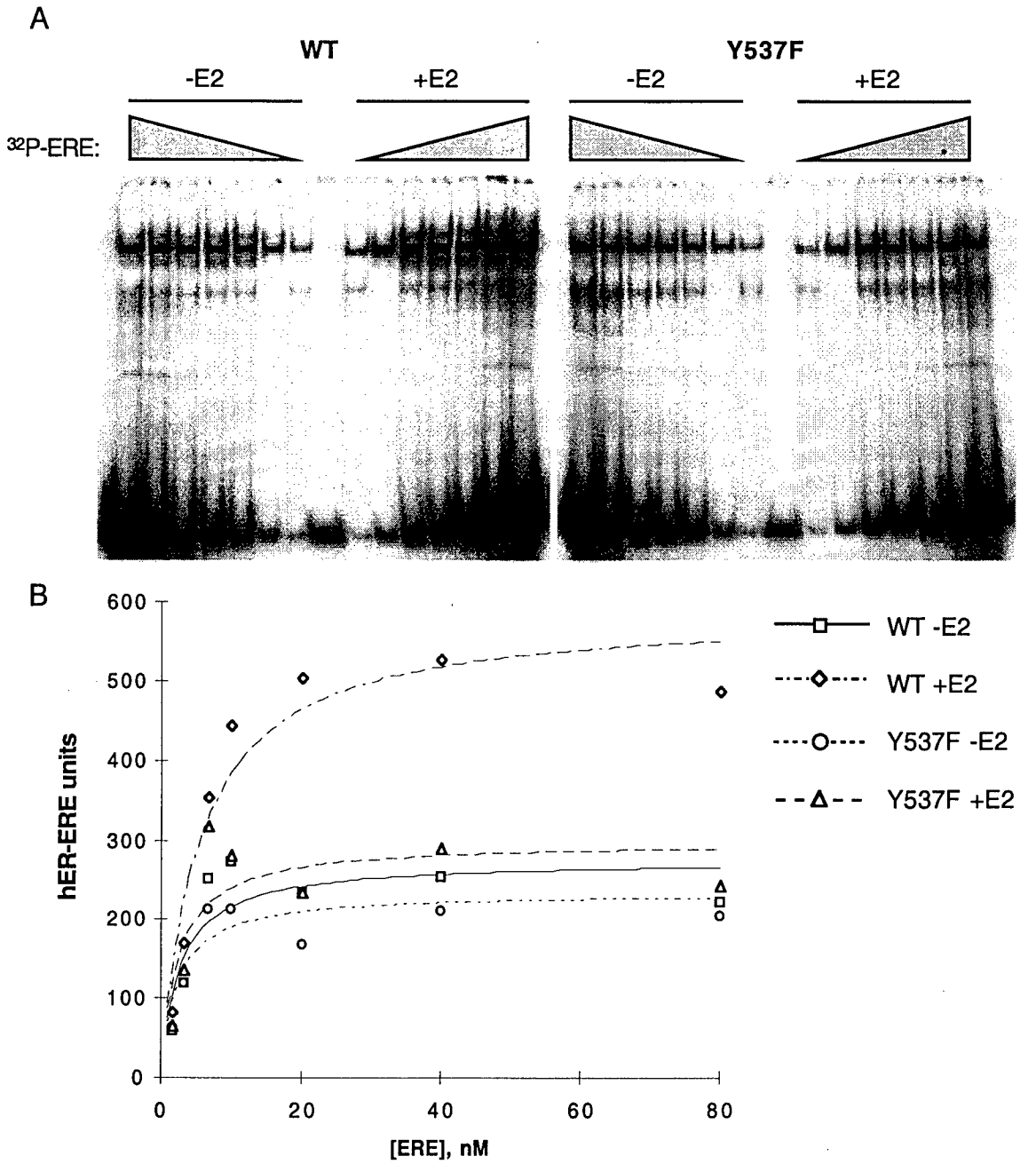
A
WT
Y537F
-E2
+E2
-E2
+E2
32P-ERE:
B
hER-ERE units
600
500
400
300
200
100
0
0
20
40
60
80
[ERE], nM
WT -E2
WT +E2
Y537F -E2
Y537F +E2

FIGURE 7

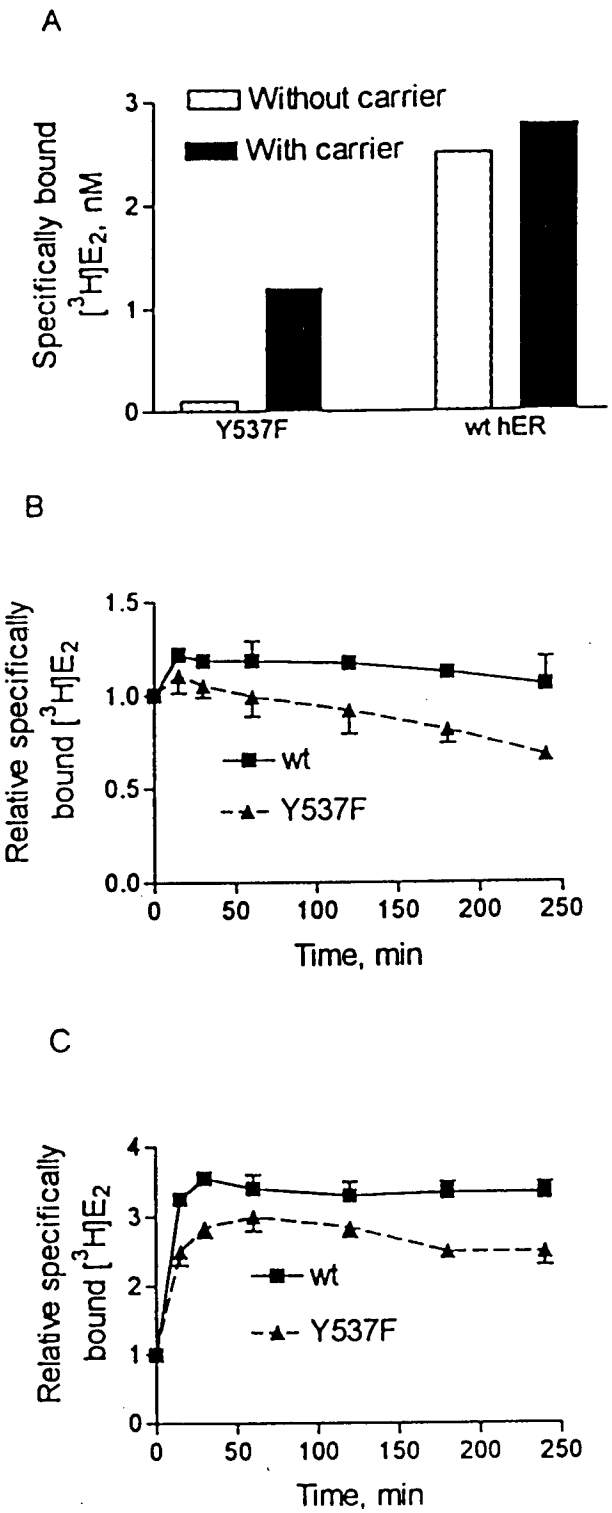
A
Without carrier
With carrier
Specifically bound [3H]E2, nM
3
2
1
0
Y537F
wt hER
B
Relative specifically bound [3H]E2
1.5
1.0
0.5
0.0
wt
Y537F
0
50
100
150
200
250
Time, min
C
Relative specifically bound [3H]E2
4
3
2
1
0
wt
Y537F
0
50
100
150
200
250
Time, min

FIGURE 8

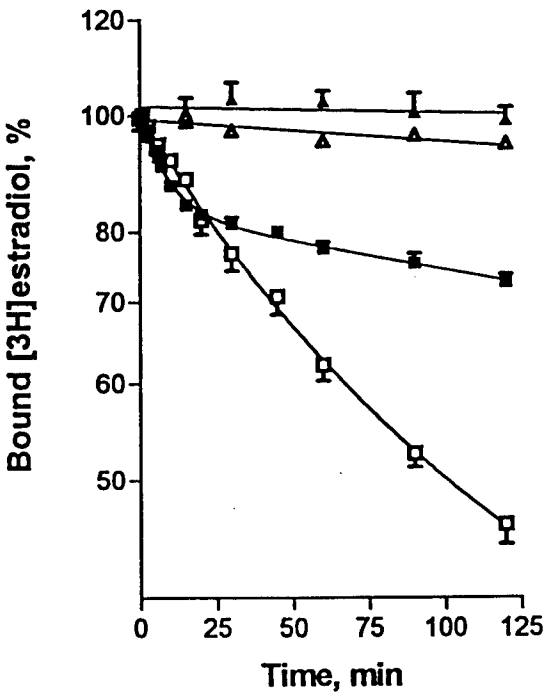
120
100
80
70
60
50
Bound [3H]estradiol, %
0
25
50
75
100
125
Time, min

FIGURE 9

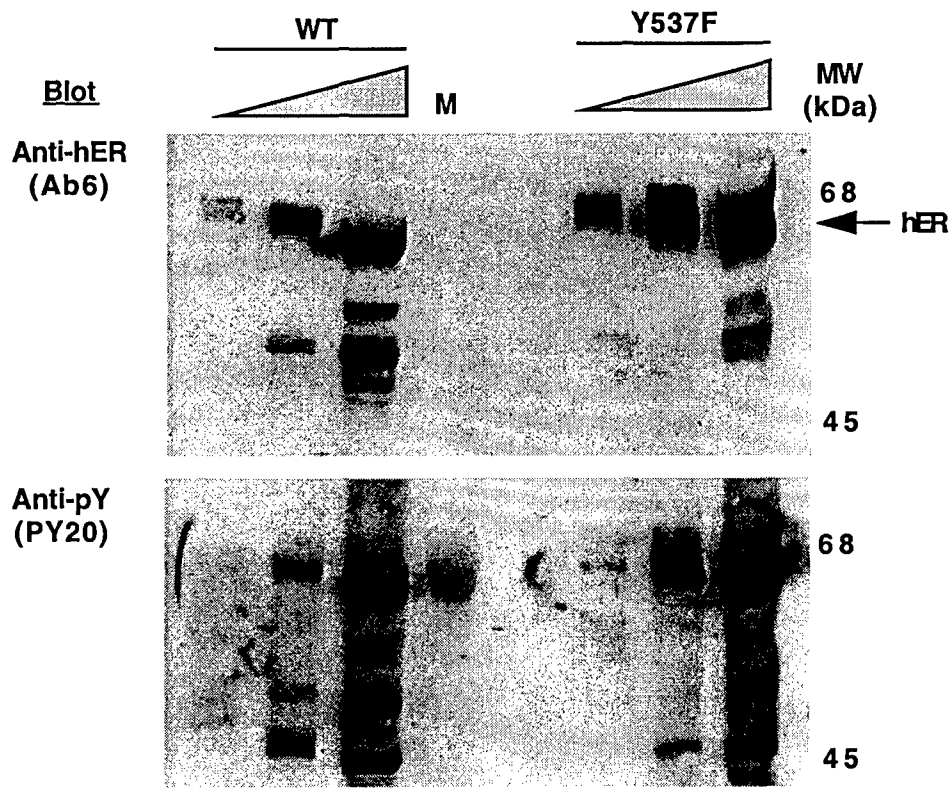

FIGURE 10

FIGURE 11

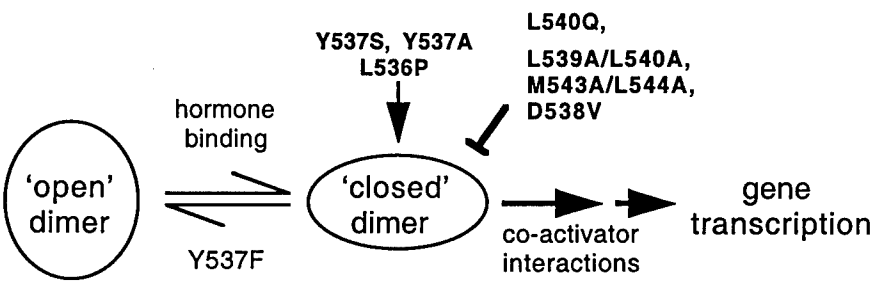
Y537S, Y537A
L536P
L540Q,
L539A/L540A,
M543A/L544A,
D538V
hormone
binding
'open'
dimer
'closed'
dimer
gene
transcription
co-activator
interactions
Y537F

Table 1. Summary of hER Y537 and Helix 12 Mutations and Their Effects on Hormone Induced Transcription and Observed Phenotype

hER	transcriptional response	phenotype	ref.
wt	hormone dependent	wild type protein	
Y537F	hormone dependent	decreased stability	10-16
L540Q	hormone dependent	dominant negative	26,27
Y537S Y537A Y537E Y537D Y537N L536P	hormone independent	constitutively active	14-18
L539A/ L540A M543A/ L544A	none	transactivation (AF-2) deficient	47-49
D538V	hormone dependent	reduced response	51

Receptors were studied in various systems. Refer to the indicated references for each mutant. The Y537 mutants display various degrees of constitutive activity. Where appropriate, mouse ER residues are indicated as the corresponding human sequences.

Preventing Estrogen Receptor Action with Dimer-Interface Peptides*

DAMD17-96-1-6219
Supplemental Material

Manuscript 2

Matthew R. Yudt and Shohei Koide[‡]

Department of Biochemistry and Biophysics

University of Rochester School of Medicine and Dentistry

Rochester, New York 14642

Running title: Estrogen Receptor Interface Peptides

Keywords: helical peptides, protein dimerization, protein aggregation, rational design.

SUMMARY

The human estrogen receptor (hER) is a ligand-activated transcription factor which functions as a homodimer. We sought to further understand the molecular processes involved in dimerization, and to develop a reagent that may function as an antiestrogen independent of the ligand binding site. To this end, we designed a 16 residue 'dimer-interface' oligopeptide derived from the helical region of the hER which is directly involved in dimerization. This peptide, termed the I-box peptide, has a high helical propensity in aqueous solution. The I-box peptide blocks hER action by causing aggregation and precipitation of both the ligand-bound and apo-hER. This effect is dependent on the helical nature of the peptide. A single Ile to Pro mutation in the helical region of the I-box peptide significantly reduces the helical content and abolishes the precipitation activity. Furthermore, the peptide activity appears to be specific for the hER. The I-box peptide does not significantly affect other proteins or steroid receptors tested. A homologous peptide derived from the nuclear receptor RXRα dimer interface, and a LXXLL-containing peptide from the coactivator TIF2 have no detectable *in vitro* effect on hER function or solubility. Our data suggest that rationally designed molecules capable of affecting steroid receptor quaternary structures may be potential avenues for the development of specific inhibitors of this class of proteins.

INTRODUCTION

The estrogen receptor-α (hER)[1] belongs to a superfamily of ligand-activated transcription factors (1). These proteins, most of which function as homo- or heterodimers, are characterized by three functional domains. A centrally located, highly conserved, DNA binding domain (DBD) is flanked by a variable amino-terminal domain containing the transcriptional activation function 1 (AF-1), and a carboxy-terminal ligand binding domain (LBD) which harbors a second transcriptional activation function (AF-2) and a large dimerization interface (2-4). The crystal structures of both the DBD and LBD have been determined for the ER and a number of other superfamily members (5-9). A mechanism for the transactivation and antagonism of the hER has emerged in the last two years based on these atomic structures. In this model, transactivation is accomplished through an agonist-induced protein interaction with coactivator proteins (10). The structures have revealed that the ligand-dependent AF-2 consists of the protein surface generated upon agonist binding, primarily through a helix 12 conformational change which allows the interaction with coactivators (8,11). Remarkably, the binding of antagonists distorts this coactivator binding surface by causing a realignment of helix 12, thereby inhibiting the LBD-coactivator interaction. These models agree with the available physiological and biochemical data regarding antiestrogen action (12,13).

In addition to its role in the female reproductive system, the hER regulates the growth and differentiation of skeletal, neural, and cardiovascular tissues in both males and females (14). The estrogen receptor is also a therapeutic target of, and a clinical marker for, estrogen-responsive breast tumors (15). A diverse group of ligands exist which modulate hER transcriptional activation and the physiological response of estradiol. For example, the active metabolite of the nonsteroidal antiestrogen tamoxifen, 4-hydroxy tamoxifen (4-OHT), antagonizes hER function in the breast and, when used clinically, results in remission of nearly 50% of all hER-positive breast tumors (16). However, the drawbacks of tamoxifen use include an increased risk of endometrial and uterine cancers. Moreover in many cases, ER-positive breast tumors develop a resistance to tamoxifen therapy and 4-OHT itself becomes estrogenic in these tumors (17). Synthetic hER

ligands, such as ralixofene and ICI 182,780, collectively referred to as selective estrogen receptor modulators (SERMs), display tissue specific agonist and antagonist properties. Several of these compounds, all of which bind to the estrogen binding pocket, are currently undergoing clinical trials for the treatment and prevention of breast cancer, osteoporosis and cardiovascular disease (18). However, because of the lack of a mechanistic understanding regarding the acquisition of tamoxifen resistance (19), a similar uncertainty may be faced when designing novel compounds targeted to, or depending on, hormone binding. Thus, the development of antiestrogenic reagents, which function independent of the ligand binding site, could provide an alternative strategy to treat these tumors.

The application of synthetic peptides to disrupt protein-protein interactions has been successfully demonstrated for a number of proteins. It has been assumed that a suitably designed peptide can mimic the interface of a specific quaternary structure, and thereby inhibit the biological function. Peptides have been used to block the enzymatic activity of the herpes simplex virus ribonucleotide reductase by interfering with subunit interactions (20). Other examples include the inhibition of HIV-1 reverse transcriptase and HIV-1 protease using peptides as protein interface inhibitors (21,22). Recently, a 20 amino acid helical peptide was demonstrated to effectively inhibit the enzymatic activity of thymidylate synthase by promoting specific protein aggregation (23). Similarly, Arnold and Notides reported that a tyrosine phosphorylated peptide derived from the hER helix 12 region (amino acids 532-543) is capable of blocking DNA binding activity of the hER in gel shift assays (24). Although the inhibition of DNA binding activity suggests that this peptide disrupts dimerization of the hER, the precise mechanism of the inhibitory effect is still unclear.

In this work, we designed a peptide based on the structure of the hER dimerization interface. This peptide, termed the I-box peptide, specifically precipitates the hER from cell extracts. The ability of this peptide to block hER function is dependent on its helical nature. Our results suggest that rationally designed peptides, or peptidomimetics, may be used to prevent hER activity by novel mechanisms independent of the ligand binding site.

EXPERIMENTAL PROCEDURES

Chemicals and Materials – The radioisotopes used in these studies were purchased through NEN, DuPont (Boston, MA): [^{3}H]17-β-estradiol (50 Ci/mmol); [^{3}H]R5020 (50 Ci/mmol); [^{32}P]γ-ATP (3000 Ci/mmol). Unlabeled estradiol-17β and 4-hydroxy tamoxifen were obtained from Sigma. The ICI 182,780 was kindly provided by Dr. A. E. Wakeling (Zeneca Pharmaceuticals, Mereside, UK). The peptides used in these studies were made by SynPep Corp. (Dublin, CA) using standard F-moc synthesis protocols. Peptides were purified using HPLC to greater than 90% purity and their identity was confirmed by mass spectroscopy. The anti-hER polyclonal antibody 6, directed against residues 259-278, was described previously (25). The anti-androgen receptor (hAR) antibody was a gift from Dr. C. Chang (University of Rochester, 26). The anti-progesterone receptor (hPR) antibody, AB52, was kindly provided by Dr. D. Edwards (University of Colorado). Cell culture media were purchased from Gibco. Purified hER (approximately 80% purity) was purchased from PanVera corporation (Madison, WI).

Cell Culture and Protein Synthesis - The bacculovirus-expressed hER was prepared as previously described (25). In most cases, *Sf9* whole cell extracts were used for hormone binding assays, western blots, and gel shift assays. The native hER, extracted from MCF-7 cells (27), was used in several experiments to compare with the results obtained using the recombinant receptor. For hER protein extraction, cell pellets (*Sf9* or MCF-7) were suspended in a hypotonic buffer (20 mM Tris, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1 mM Na_3VO_4, 0.5 mM PefaBlock (Boehringer Manheim), and 0.2 mM leupeptin, pH 7.4) and lysed by three freeze/thaw cycles. Potassium chloride was then added to a final concentration of 500 mM and extracts were incubated on ice for an additional 30 minutes before centrifugation at 140,000 X g. The concentration of active hER, based on estrogen binding assays, was determined by the charcoal-coated dextran method. Cell extracts, containing recombinant hPR, were also similarly prepared in a bacculovirus system. Extracts of the LNCaP prostate cancer cell line were kindly provided by Dr. C. Chang and used as a source of soluble androgen receptor and (28).

Circular dichroism spectroscopy - Circular dichroism (CD) spectra were recorded on a Jasco J720 spectropolarimeter. Data were recorded in the step scan mode, where data are averaged over a time interval of 0.25 seconds at wavelength intervals of 1.0 nm. The excitation band width was 1.0 nm. All measurements were performed using a quartz cuvette with a 0.1 cm pathlength . The sample temperature was not actively controlled, but remained stable at room temperature over the duration of the experiments. Peptide concentrations were 100 μM in PBS, pH 7.4 and contained 1% DMSO and the indicated amount of trifluoroethanol (TFE). Each spectrum was an average of 5 measurements which were subsequently smoothed. The mean molar ellipticity was determined from the relation

$$[\theta] = \theta / (10\, c\, n\, l)$$

where θ is the ellipticity (mdeg, raw data), c is the molar concentration of peptide, n is the number of amino acids in the peptide, and l is the path length (0.1 cm). The helical content was estimated from the mean residue ellipticity at 222 nm using the equation

$$\% \text{ helix} = [\theta]_{222} \text{ X } 100 / [39{,}500(1\text{-}2.57/n_p)]$$

where $[\theta]_{222}$ is the mean residue ellipticity at 222 nm and n_p is the number of peptide bonds (29).

DNA binding assay - Gel mobility shift assays (GMSA) were performed with 6 nM hER receptor extracts in binding buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 0.2 mM Na_3VO_4) and 1 mM leupeptin and 0.5 mM PefaBlock. The final salt concentration was maintained at 100 mM by the appropriate addition of a 4M KCl stock solution. Similarly, total protein concentrations of 0.2 mg/ml were achieved using 10 mg/ml insulin. Non-specific DNA binding was prevented by a 10-15 minute pre-incubation with 1 μg of poly(dI-dC) (Pharmacia Biotech.). A double stranded 27-base pair probe (5'-GATCCTAGAGGTCACAGTGACCTACGA-3'), encoding the Xenopus vitellogenin A2 ERE, was ^{32}P-end-labeled with T4 polynucleotide kinase and added to the final reaction (1-10 fmoles = 20,000 cpm). Following a one hour incubation on ice, the samples were electrophoresed on a 5% non-denaturing polyacrylamide gel for 2.5 hours, at 175 V, 4 °C, in a

0.5X TBE running buffer. The gels were dried and autoradiographs were obtained using PhosphorImager plates. Band intensities were quantified using the ImageQuant software (Molecular Dynamics).

Hormone binding assay - Cell extracts, prepared as described above, were diluted in TDGK buffer (40 mM Tris-HCl, pH 7.4, 1 mM DTT, 15% glycerol, and 150 mM KCl with 0.2 mM PefaBlock and 0.5 mM leupeptin). Ovalbumin was added to achieve a final protein concentration of 5 mg/ml. To determine the hER concentrations in cell extracts, the receptor preparation was incubated with various concentrations of [^{3}H]estradiol-17β (0.3 - 100 nM) for 16 hr on ice. Non-specific binding was measured by a parallel incubation including a 150-fold molar excess of radioinert estradiol. The unbound hormone was removed by incubation with dextran-coated charcoal (DCC) solution (0.03%/0.3% final concentrations). Following liquid scintillation counting, the specific binding was obtained by subtracting non-specific binding from total binding.

Western Blots - Whole cell extracts containing ER, PR or AR were analyzed for the respective protein by western blotting with the appropriate antibodies. Extracts were separated on 10% SDS-PAGE and transferred to polyvinylidendiflouride (PVDF) membranes. Blotting was carried out as previously described (30) using the ECL detection system.

RESULTS

Peptide design

The three dimensional structure of the hER LBD reveals a large dimer interface (8,9,31). Contacts between the monomers are made primarily through mutual interactions of the N-terminal portion of helix 10/11 (amino acids 505-515) but also involves interactions between helix 8 from one monomer and helices 9 and 10/11 from the neighboring monomer (Fig. 1A and B). It should be noted that, in the hER LBD structure, the 10th and 11th helices are one contiguous helix, and not two separate helices as in the RXR LBD (6). Thus, we denote this contiguous helix as helix 10/11 in this article. These structural observations agree with various reports on the location of a dimerization interface within the hER and other nuclear hormone receptor LBDs (3,32). A sequence alignment of the carboxy terminal region of various nuclear receptor LBDs is shown in Figure 1D. The helix 10/11 region, which contains the ninth heptad repeat in the hER (33), has also been referred to as the identity box in RXR, or I-box, because of its role as a heterodimerization interface (34,35).

Arnold and Notides demonstrated that a 12 amino acid phosphotyrosine peptide (Yp537), corresponding to hER residues 532-542 (helix 12), was able to inhibit specific DNA binding of the hER (24). Further analyses have revealed that the leucine-rich region of the Yp537 peptide was necessary for the inhibitory effect (Yudt and Notides, unpublished data). The Yp537/helix 12 region has striking homology to the helix 10/11 region involved in hER dimerization. Therefore, we hypothesized that the Yp537 peptide may function as a 'dimerization inhibitor' by mimicking the dimerization interface. In order to test this hypothesis, we examined the effect of peptides directly derived from the helix 10/11 region. We chose a segment corresponding to residues 503-518 that contains a majority of helix 10/11 and is centrally located in the dimer interface (Fig. 1C).

The I-box peptide is helical

Using the sequence-based algorithm AGADIR (36), a peptide corresponding to the hER helix 10/11 region from residues R503 to S518 (hER "I-box" peptide) was predicted to contain

significant helical content at pH 7.4 and an ionic strength of 150 mM. In contrast, a control peptide with a proline in place of the isoleucine corresponding to position 510 was predicted to contain essentially no helical propensity. Circular dichroism spectroscopy was used to assess the actual secondary structure content of these I-box peptides. Figure 2A shows the CD spectra of the I-box peptide (IB-WT) at increasing trifluoroethanol (TFE) concentrations. The spectral shapes are typical of an alpha helix as indicated by minima at 208 nm and 222 nm. On the other hand, at low TFE concentrations the proline containing peptide (IB-PRO) has a significantly smaller ellipticity at 222 nm and also exhibits negative ellipticity below 205 nm, indicative of a random coil. TFE titration of IB-PRO induces a helical structure and an isodichroic point near 203 nm is an indication of coil/helix transitions. For both peptides, the ellipticity at 222 nm exhibits a sigmoidal dependence on the TFE concentration, suggesting that helix formation is a cooperative process (Figure 2B). However, the amount of helix is significantly larger in the IB-WT relative to IB-PRO peptide under all conditions. The observed helicity of IB-WT at 1% TFE is linear over a range of concentrations (10-100 μM), suggesting that the quaternary (oligomerization) state of the peptide does not change in this concentration range (37). All of the peptides used in these studies are soluble at least up to 100 μM in aqueous buffer solutions.

The I-box peptide specifically precipitates the hER

The effects of the IB peptides on hER activity were then investigated. Both the recombinant receptor from baculovirus-infected Sf9 cells (25) and the native hER from the MCF-7 breast cancer cell line were used in these studies. Following incubations with peptides, the soluble cell extracts were centrifuged and supernatants analyzed for hER content by western blotting. In the presence of IB-WT the unliganded hER precipitated upon centrifugation (Fig. 3A). Further analysis of other proteins present in the cell extracts revealed that precipitation of the hER was specific, and no other protein bands appeared to be affected as drastically as the hER by IB-WT peptide incubations (Fig. 3B). The IB-WT also precipitated the purified hER, but not carrier proteins (bovine serum albumin or insulin, data not shown), indicating that the effect is specific for

the hER and that precipitation does not require the presence of unidentified intermediary factors. The IC_{50} of the IB-WT peptide for hER precipitation, measured as a percent of total soluble hER, is approximately 5 μM. In contrast to these observations with the IB-WT peptide, the hER remains soluble after incubation with the IB-PRO peptide or the Yp537 peptide (Fig. 3A). These results suggest that precipitation of the hER with a dimer-contact peptide is dependent on both the sequence and the secondary structure of the peptide. Gel mobility shift analysis shows that peptide-induced hER aggregates do not bind the estrogen response element (ERE) (Fig. 3C). Furthermore, during gel shift analysis, we did not observe a decrease in level of free ERE probe or the presence of a large aggregate in the gel following incubations with the IB-WT. Dilution experiments, in which an overnight incubation of the hER with 20 μM IB-WT peptide was diluted to less than one micromolar resulted in recovery of hER DNA binding activity (data not shown).

Next, the effects of common hER ligands on IB-WT induced precipitation were examined. The precipitation effect of IB-WT observed above was not altered when the hER was pre-bound with estradiol-17β, 4-OHT, or ICI 182,780 (figure 4, lane 2). To test the effect of IB-WT peptide on the ligand-bound hER, the receptor was labeled with [^{3}H]estradiol-17β prior to the addition of peptides. Remarkably, the precipitated hER remained in a ligand bound state (Fig. 5A), suggesting that the LBD of the precipitated receptor retains a native-like conformation. However, several attempts to recover ligand binding activity from the precipitated apo-receptors were unsuccessful. These results suggest that precipitation of the hER is independent of the hormone binding pocket, and the large conformational changes of the LBD which occur upon agonist/antagonist binding do not significantly alter the effectiveness of the IB-WT peptide.

Specificity of the IB-hER interaction

A comparison of the helix 10/11 regions of nuclear receptors (Fig. 1B) shows the highest homology between the hER IB-WT sequence and the same region of RXRα. A 16 residue peptide was prepared which corresponds to the RXRα H10 region (IB-RXR: RXRα amino acids 414-430). The IB-RXR peptide had little effect on the hER (Fig. 4, lane 5). Circular dichroism of the

IB-RXR at low TFE concentrations was similar to the IB-PRO spectra shown in Figure 2A. These results are not surprising considering the presence of a proline in the IB-RXR peptide and consequently a low helical content predicted by AGADIR. A shorter hER I-box peptide, IB-WT10, was prepared which corresponds to hER amino-acids 503-512, in which the C-terminal 6 residues of IB-WT were removed. Residues 503-512 are predicted to form the helical core of the longer IB-WT peptide. Interestingly, the shorter peptide (IB-WT10) precipitated the hER similarly to the IB-WT (Fig. 4, lane 3), although the IC_{50} was increased nearly two-fold (not shown). The specificity of the IB-WT peptide on other steroid receptor superfamily members was tested using the progesterone receptor (hPR) and the androgen receptor (hAR) which were readily accessible to us. The IB-WT peptide had no detectable effect on recombinant hPR activity or solubility (Fig. 5B). A western blot of the supernatants from hPR-peptide incubations confirmed that the hPR remained soluble following incubation with the IB-WT peptide (Fig. 6A). Similarly, the hAR was not precipitated from cell extracts upon IB-WT incubation (Fig. 6B). These results suggest that the IB-WT effect on hER function is receptor-specific.

DISCUSSION

In this report we demonstrate that a dimer-interface peptide, derived from the helical region of the hER protein involved in homodimerization (residues 503-518), can specifically block receptor function by causing aggregation and precipitation of the hER from solution. Although the precipitated hER appears to retain bound estradiol, it is unable to bind DNA in gel shift assays. The inhibitory effect of the peptide is specific for the hER and no other proteins appear to be precipitated by the peptide. In addition, the peptide precipitated the purified hER, suggesting that the inhibitory effect is due to direct interactions between the peptide and the hER. We have shown that the helical structure of the IB-WT peptide is correlated with the ability to precipitate the hER.

It was previously demonstrated that a 22-amino acid region, encompassing hER residues 497-518, was capable of restoring DNA-binding activity to dimerization defective (truncated) murine ER mutants (38). This fragment contains the I box sequence and a synthetic peptide corresponding to this sequence was further characterized and found to possess a propensity for α-helical conformation, but did not itself dimerize below millimolar concentrations (39). However, those studies did not investigate the effect of the free peptide on hER function. At this point in time, the mechanism of peptide induced precipitation is unclear. There are several possible mechanisms. By mimicking the dimer interface, the peptide-hER interaction may result in partial misfolding by exposing normally buried hydrophobic regions of the hER, thus promoting aggregation. Notably, several mutations within the I-box sequence of the hER (containing the ninth heptad repeat) are presumed to result in dimerization deficient mutants, based on their inability to bind ERE or co-precipitate with the wild-type hER under selective immunoprecipitation assays (3). However, none of these 'dimerization-deficient' mutants are reported to precipitate or form insoluble aggregates, and all maintain hormone binding activity. Thus, it is not clear why disruption of the hER dimer interface with the IB peptide would lead to precipitation from whole cell extracts. The molar ratio of peptide to hER in our experiments is greater than one thousand, and the stoichiometry of peptide-hER complex(es) is not yet known. Furthermore, the solubility of the IB-WT peptide in aqueous buffer is limited to about 100 μM. In this respect, the IB-WT

peptide may serve as a 'molecular glue', promoting the association and aggregation of hER molecules.

According to AGADIR predictions, the leucine-rich region at the amino terminal end of the peptide is important for the helical propensity of the I-box peptide. The interaction of the hER LBD with transcriptional co-activators is mediated by leucine-rich motifs within the co-activator (LXXLL). Interestingly both the hER dimer interface (H10/11) and the AF-2 AD core region (helix 12) contain this LXXLL motif. However, beyond the leucine spacing the homology between the coactivator interaction motifs and the two hER LXXLL sequences is low. The hER is unique in that it is the only nuclear receptor (of those shown in Fig. 1B) to contain the LXXLL sequence in either the dimer interface or the helix 12 AF-2 AD core region. The peptides used in this study all contain LXXLL motifs (except IB-RXR) but only the IB-WT and IB-WT10 effectively precipitate the hER. The IB-WT peptide contains the highest homology with the I-box core region of RXRα. However, the corresponding peptide (IB-RXR) has a low helical propensity and no observable effect on hER function.

The hER IB-WT peptide had no effect on either hPR or hAR solubility. The hPR and hAR sequences are nearly identical at the dimer interface (helix 10) and diverge considerably from the corresponding ER sequence in both hydrophobic and charged residues (Fig. 1D). In addition to the sequence differences of the helix 10/11 region, the IB-WT specificity for the hER may be explained in light of the structural differences in the dimer interface between the hPR and hER (31). Although the overall folding of the LBDs are similar between the hPR and other nuclear receptor structures, the dimerization interface observed for the hPR is less than half as a large as that for the hER and RXR proteins, and involves different regions of the receptor (40).

Although precipitation may not appear as an ideal strategy for *in vivo* inhibition of the hER, it is important to realize that misfolded or aggregated proteins are usually degraded *in vivo*. Also, the hER, like other steroid hormone receptors, is associated in the cell with chaperones such as hsp90 and hsp70 (41,42). These chaperone proteins prevent aggregation by binding nascent polypeptides and misfolded proteins. Thus, the conditions *in vivo* may not favor peptide-induced

aggregation. However, the binding of the IB-WT peptide to the hER may be sufficient for preventing the hER function *in vivo*. Studies are underway investigating the *in vivo* efficacy of the IB-WT peptide as a novel antiestrogen. Future studies are also aimed at determining the precise peptide interaction region on the hER and understanding the mechanism of precipitation *in vitro*. The potential to block receptor function, independent of the active site of hormone binding, raises new avenues for drug design and discovery.

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REFERENCES

1. Evans, R. M. (1988) *Science* **240,** 889-895
2. Kumar, V., Green, S., Stack, G., Berry, M., Jim, J.-R., and Chambon, P. (1987) *Cell* **51,** 941-951
3. Fawell, S. E., Lees, J. A., White, R., and Parker, M. G. (1990) *Cell* **60,** 953-62
4. Danielian, P. S., White, R., Lees, J. A., and Parker, M. G. (1992) *EMBO J.* **11,** 1025-33
5. Schwabe, J. W. R., Chapman, L., Finch, J. T., and Rhodes, D. (1993) *Cell* **75,** 567-578
6. Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995) *Nature* **375,** 377-82
7. Wurtz, J. M., Bourguet, W., Renaud, J. P., Vivat, V., Chambon, P., Moras, D., and Gronemeyer, H. (1996) *Nat. Struct. Biol.* **3,** 87-94
8. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95,** 927-937
9. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389,** 753-8
10. Jenster, G. (1998) *Mol. Cell. Endocrinol.* **143,** 1-7
11. Nolte, R. T., Wisely, G. B., Westin, S., Cobb, J. E., Lambert, M. H., Kurokawa, R., Rosenfeld, M. G., Willson, T. M., Glass, C. K., and Milburn, M. V. (1998) *Nature* **395,** 137-143
12. Notides, A. C., and Nielsen, S. (1974) *J. Biol. Chem.* **249,** 1866-73
13. Pasqualini, J. R., Sumida, C., and Giambiagi, N. (1988) *J. Steroid Biochem.* **31,** 613-43
14. Korach, K. (1994) *Science* **266,** 1524-1527
15. Jordan, V. C., Jeng, M. H., Jiang, S. Y., Yingling, J., and Stella, A. L. (1992) *Seminars in Oncology* **19,** 299-307
16. Nayfield, S. G. (1995) *J. Cell. Biochem.* **S 22,** 42-50
17. Gottardis, M. M., Robinson, S. P., Satyaswaroop, P. G., and Jordan, V. C. (1988) *Cancer Res.* **48,** 812-5

18. Jordan, V. C. (1998) *J. Natl. Cancer Inst.* **90,** 967-971

19. Bilimoria, M. M., Assikis, V. J., Muenzner, H. D., Wolf, D. M., Satyaswaroop, P. G., and Jordan, V. C. (1996) *J. Steroid Biochem. Mol. Biol.* **58,** 479-88

20. Dutia, B. M., Frame, M. C., Subak-Sharpe, J. H., Clark, W. N., and Marsden, H. S. (1986) *Nature* **321,** 439-441

21. Divita, G., Restle, T., Goody, R. S., Chermann, J.-C., and Baillon, J. G. (1994) *J. Biol. Chem.* **269,** 13080-13083

22. Schramm, H. J., Boetzel, J., Buttner, J., Fritsche, E., Gohring, W., Jaeger, E., Konig, S., Thumfart, O., Wenger, T., Nagel, N. E., and Schramm, W. (1996) *Antiviral Res.* **30,** 155-170

23. Prasana, V., Bhattacharjya, S., and Balaram, P. (1998) *Biochemistry* **37,** 6883-6893

24. Arnold, S. F., and Notides, A. C. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92,** 7475-9

25. Obourn, J. D., Koszewski, N. J., and Notides, A. C. (1993) *Biochemistry* **32,** 6229-36

26. Kimura, N., Mizokami, A., Oonuma, T., Sasano, H., and Nagura, H. (1993) *J. Histochem. Cytochem.* **41,** 671-8

27. Green, S., Walter, P., Greene, G., Krust, A., Goffin, C., Jensen, E., Scrace, G., Waterfield, M., and Chambon, P. (1986) *J. Steroid Biochem.* **24,** 77-83

28. Mizokami, A., Yeh, S. Y., and Chang, C. (1994) *Mol. Endocrinol.* **8,** 77-88

29. Chen, Y. H., Yang, J. T., and Chau, K. H. (1974) *Biochemistry* **13,** 3350-9

30. Denton, R. R., Koszewski, N. J., and Notides, A. C. (1992) *J. Biol. Chem.* **267,** 7263-8

31. Tanenbaum, D. M., Wang, Y., Williams, S. P., and Sigler, P. B. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 5998-6003

32. Jin, C. H., Kerner, S. A., Hong, M. H., and Pike, J. W. (1996) *Mol. Endocrinol.* **10,** 945-57

33. Forman, B. M., Yang, C.-R., Au, M., Casanova, J., Ghysdael, J., and Samuels, H. H. (1989) *Mol. Endocrinol.* **3,** 1610-1626

34. Perlmann, T., Umesono, K., Rangarajan, P. N., Forman, B. M., and Evans, R. M. (1996) *Mol. Endocrinol.* **10,** 958-66

35. Lee, S. K., Na, S. Y., Kim, H. J., Soh, J., Choi, H. S., and Lee, J. W. (1998) *Mol. Endocrinol.* **12,** 325-32

36. Munoz, V., and Serrano, L. (1994) *Nat. Struct. Biol.* **1,** 399-409

37. Soler-Gonzalez, A. S., and Fersht, A. R. (1997) *Eur. J. Biochem.* **249,** 724-32

38. Lees, J. A., Fawell, S. E., White, R., and Parker, M. G. (1990) *Mol. Cell. Biol.* **10,** 5529-31

39. Baumann, H., and Hard, T. (1995) *Eur. J. Biochem.* **230,** 879-885

40. Williams, S. P., and Sigler, P. B. (1998) *Nature* **393,** 392-6

41. Chambraud, B., Berry, M., Redeuilh, G., Chambon, P., and Baulieu, E.-E. (1990) *J. Biol. Chem.* **265,** 20686-20691

42. Landel, C. C., Kushner, P. J., and Greene, G. L. (1994) *Mol. Endocrinol.* **8,** 1407-19

43. Kraulis, P. (1991) *J. Appl. Cryst.* **24,** 946-950

44. Nicholls, A., Sharp, K. A., and Honig, B. (1991) *Proteins* **11,** 281-296

FOOTNOTES

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Dedication: This manuscript is dedicated to the late Professor Angelo C. Notides.

‡To whom correspondence should be addressed: Department of Biochemistry and Biophysics, Box 712, University of Rochester, School of Medicine and Dentistry, Rochester, New York 14642. Email: Shohei_Koide@urmc.rochester.edu

[1]The abbreviations used are: hER, human estrogen receptor (note that there are two forms of the estrogen receptor, alpha and beta, and that hER denotes the alpha form which was used in the studies described here); ERE, estrogen response element; hAR, human androgen receptor; hPR, human progesterone receptor; RXR, retinoic X receptor; AF-1, trascriptional activation function 1; AF-2, trascriptional activation function 2; DBD, DNA binding domain; LBD, ligand binding domain; IB-WT, a peptide corresponding to residues 503-518 of hER; IB-PRO, a mutant IB-WT peptide containing Ile510Pro mutation; IB-WT10, a peptide corresponding to residues 503-512; 4-OHT, 4-hydroxy tamoxifen; CD, circular dichroism; GMSA, gel mobility shift assay; TFE, trifluoroethanol.

FIGURE LEGENDS

Fig. 1. **Structure of the hER LBD.** *A*, Schematic drawing of the hER LBD dimer (9). The dimer interface is indicated with lines. Helix 10/11 is shown in red. *B*, Schematic drawing of the hER LBD monomer viewed from the dimer interface, showing the position of helix 10/11 in red. *C*, The dimer interface of the hER LBD. The molecular surface of residues 503-518 (corresponding to the I-box peptide; shown in red) is centrally located in the dimerization contact surface (shown in green). Panels A and B were made with MOLSCRIPT (43), and C with GRASP (44). *D*, A sequence alignment of the hER and other steroid and nuclear receptor ligand binding domains. The two carboxy terminal helices H10/11 and H12, which comprise the dimerization and AF-2 regions respectively, are shown. In the hER LBD structure, the 10th and 11th helices are one contiguous helix, and not two separate helices as in the RXR LBD (6). The underlined sequences denote homologous LXXLLL motifs. The shaded box represents the sequence of the I box peptide (IB-WT) used in this study. The abbreviations are: hTRβ1, human thyroid hormone receptor β1; hVDR, vitamin D receptor; hRARγ, retinoic acid recptor γ; hRXRα, retinoic X receptor α; hPR, progesterone receptor; hAR, androgen receptor; hGR, glucocorticoid receptor; and hMR, mineralocorticoid receptor.

Fig. 2. **Circular dichroism spectroscopy of designed peptides**. The IB-WT (RLAQLLLILSHIRHMS) and the IB-PRO (RLAQLLLPLSHIRHMS) peptides were dissolved in DMSO to a final stock concentration of 10 mM. The peptides were then titrated with trifluoroethanol (TFE) in PBS, pH 7.4 at a final peptide concentration of 0.1 mM and 1% DMSO. *A*, Comparison of IB-WT and IB-PRO CD spectra resulting from TFE titration. Minima at 222 nm and 208 nm with corresponding maximal ellipticity below 205 nm is characteristic of a helical structure, while negative ellipticity below 205 nm indicates a random coil. The ellipticity at 222 nm decreases with increasing TFE (1-30%), as indicated by the arrow. *B*, The percent helical contents of the two peptides are plotted against the TFE concentration. Helical content was determined as

described in *Experimental Procedures*. The θ value for 100% helix was chosen as –32,000 deg cm^2 $dmol^{-1}$ (29).

Fig. 3. **Precipitation of the hER with the IB-WT peptide**. *A*, hER-containing soluble *Sf9* cell lysates were incubated for 30 minutes on ice in the absence (C) or presence of 1 μM estradiol-17β (E2), 1% DMSO (D), 250 μM of peptide Yp537 (CNVVPLYpDLLLE)(24), and 20 μM of the IB-WT and IB-PRO peptides, respectively. Following centrifugation, the supernatants were analyzed by western blotting with an anti-hER polyclonal antibody. Only in the presence of the IB-WT peptide was the hER removed from solution upon centrifugation. *B*, Cell lysates treated as in A with DMSO, the IB-WT and IB-PRO peptides, respectively, were subjected to SDS-PAGE and proteins visualized with Coomassie blue staining. A range of total protein concentrations, corresponding to 500, 250 and 125 μg of total protein, is shown. *C*, Blocking hER DNA binding activity with the IB-WT peptide. Purified recombinant hER (6 nM) was titrated with IB-WT and IB-PRO respectively, prior to gel mobility shift assay (GMSA). Final peptide concentrations were 0, 2.5, 5, 10, and 20 μM. Non-specific DNA and ^{32}P-ERE were added following 30 minute peptide incubations. The reaction mixtures were centrifuged for 5 minutes prior to loading the gel to remove insoluble aggregates. The free ERE was not precipitated by IB-WT peptide at concentrations less than 50 μM. Data shown are representative of 3 or more separate experiments.

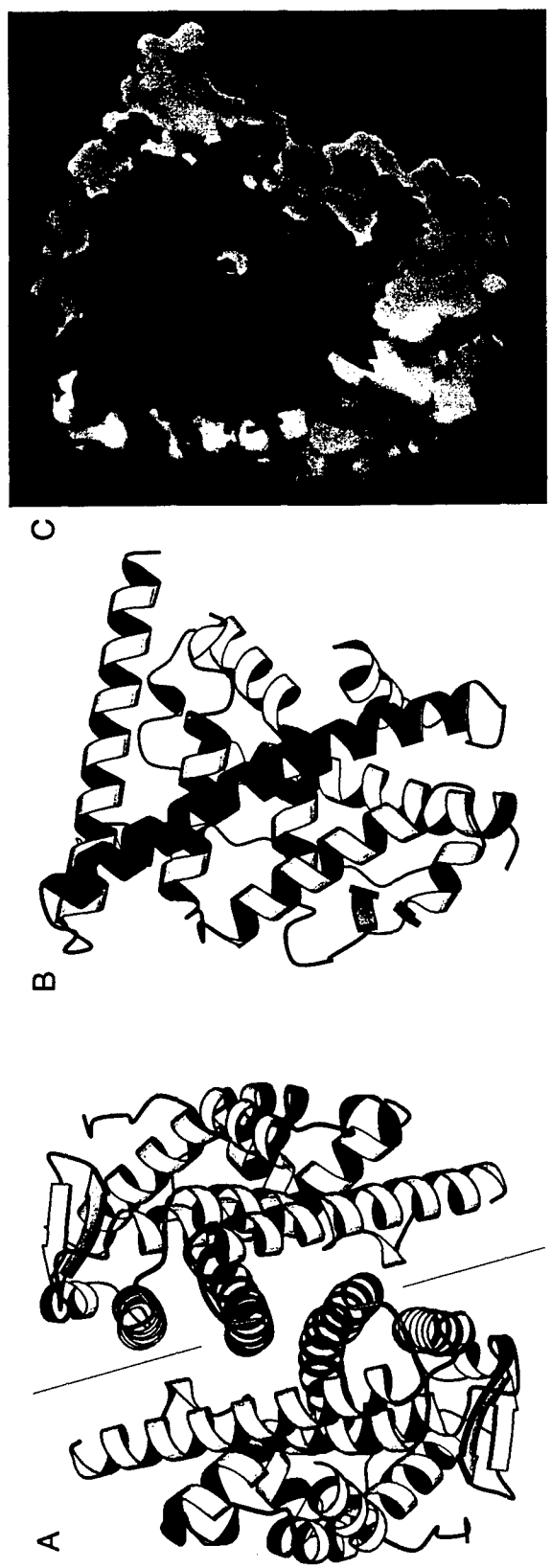
Fig 4. **Effects of hER ligands and different peptides on hER precipitation by the IB-WT peptide**. *Sf9* whole cell extracts containing approximately 120 nM recombinant hER were respectively labeled with 1 μM of estradiol-17β, 4-hydroxy tamoxifen, and ICI 182,780 overnight at 4 C. Extracts were then diluted in TDGK buffer to 10 nM hER before addition of the peptides. Following incubation for 30 minutes on ice, the extracts were centrifuged at 15,000 X g for 15 minutes. The supernatants were subjected to western blot analysis for hER content. Lane 1, 1% DMSO (control with no peptide); 2, 20 μM IB-WT; 3, 20 μM IB-WT10 (RLAQLLLILS);

4, 20 μM IB-PRO; 5, 20 μM IB-RXR (RFAKLLLRLPALRSIG); 6, 100 μM HD3 (ENALLRYLLDRD). Data shown are representative of two individual experiments.

Fig 5. **Precipitation of hER and hPR monitored by bound hormones.** A, hER-containing cell extracts were labeled with [^{3}H]estradiol-17β overnight on ice. Unbound hormone was removed with charcoal-coated dextran. Receptors were then incubated for 30 minutes on ice in the presence of 1% DMSO (no peptide), or 20 μM of the IB-WT, IB-WT10, or IB-PRO peptides, respectively. Following centrifugation, the pellets were washed once with TDGK buffer and the amount of receptor-bound [^{3}H]estradiol was measured in both the supernatant and pellet fractions. *B*, Human progesterone receptor (PR)-containing *Sf9* extracts were labeled with [^{3}H]R5020 overnight on ice and treated identically as the hER in A. The IB-WT peptide does not precipitate either [^{3}H]-estradiol or [^{3}H]R5020 under these assay conditions. Non-specific hormone binding was determined to be less than 10% of the total binding in a control experiment in which the extracts were incubated in the presence of excess unlabeled hormone. The total amount of soluble, receptor-bound hormone, prior to peptide incubation, was taken as 100% for both hER and PR. A similar precipitation pattern was observed using MCF-7 cells as the source of soluble hER (~1 nM).

Fig. 6. **Effect of I box peptides on androgen and progesterone receptors.** *A*, *Sf9* cell extracts containing unliganded progesterone receptor (hPR), and *B*, soluble LNCaP human prostate cancer cell lysates containing human androgen receptor (hAR) were treated for 30 minutes on ice with 1% DMSO (lane 1); 50 μM IB-WT (lane 2); 50 μM IB-PRO (lane 3); 50 μM IB-RXR (lane 4); and 50 μM IB-WT10 (lane 5). Following centrifugation, supernatants were analyzed by western blotting with either an anti-hPR monoclonal antibody (A) or a polyclonal anti-hAR antibody (B).

FIGURE 1



D.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hVDR    365  GSHL LYAKMIQKLADLRSLNEEHSKQYRCLSFQPECS---MKLTPLVLEVFGN  424
hRARγ   371  SQPY MFPRMLMKITDLRGISTKGAERAITLKMEIPGP-----MPPLIREMLEN  418
hRXRα   410  EQPG RFAKLLLRLPALRSIGLKCLEHIFFFKLIGDTP-----IDTFLMEMLEA  457

hER     499  QQHQ RLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVP-----LYDLLLEMLDA  546

hPR     865  SSSQ RFYQLTKLLDNLHDLVKQLHLYCLNTFIQSRAL--SVEFPEMMSEVIAA  915
hAR     851  SCSR RFYQLTKLLDSVQPIARELHQFTFDLLIKSHMV--SVDFPEMMAEIISV  901
hGR     710  QNWQ RFYQLTKLLDSMHEVVENLLNYCFQTFLDKTMS---IEFPEMLAEIITN  759
hMR     916  QSWQ RFYQLTKLLDSMHDLVSDLLEFCFYTFRESHAL--KVEFPAMLVEIISD  966
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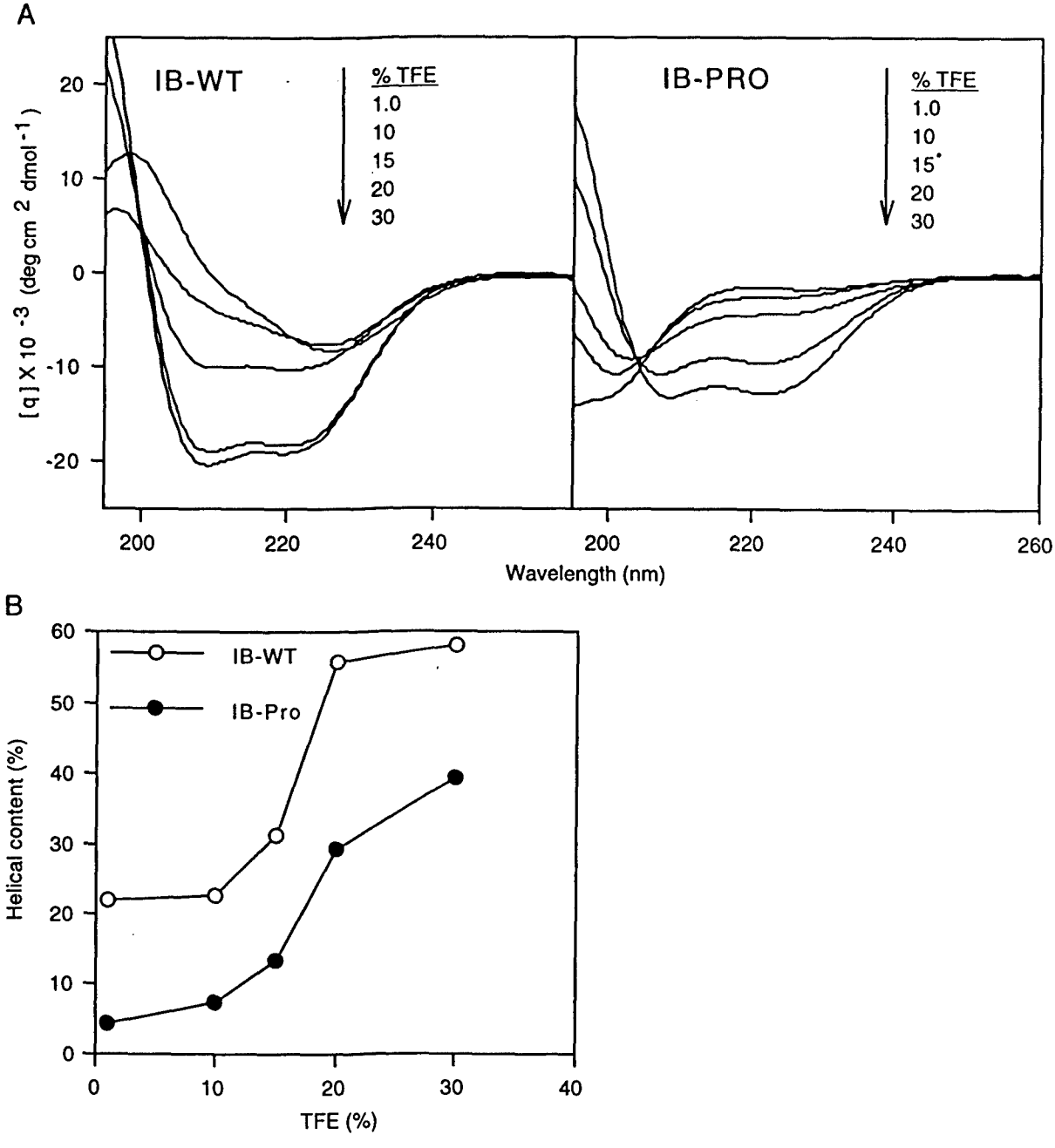
Yudt and Koide Figure 1D

FIGURE 2


A
IB-WT
% TFE
1.0
10
15
20
30
IB-PRO
% TFE
1.0
10
15
20
30
[q] X 10 -3 (deg cm 2 dmol -1)
20
10
0
-10
-20
200
220
240
200
220
240
260
Wavelength (nm)
B
IB-WT
IB-Pro
Helical content (%)
60
50
40
30
20
10
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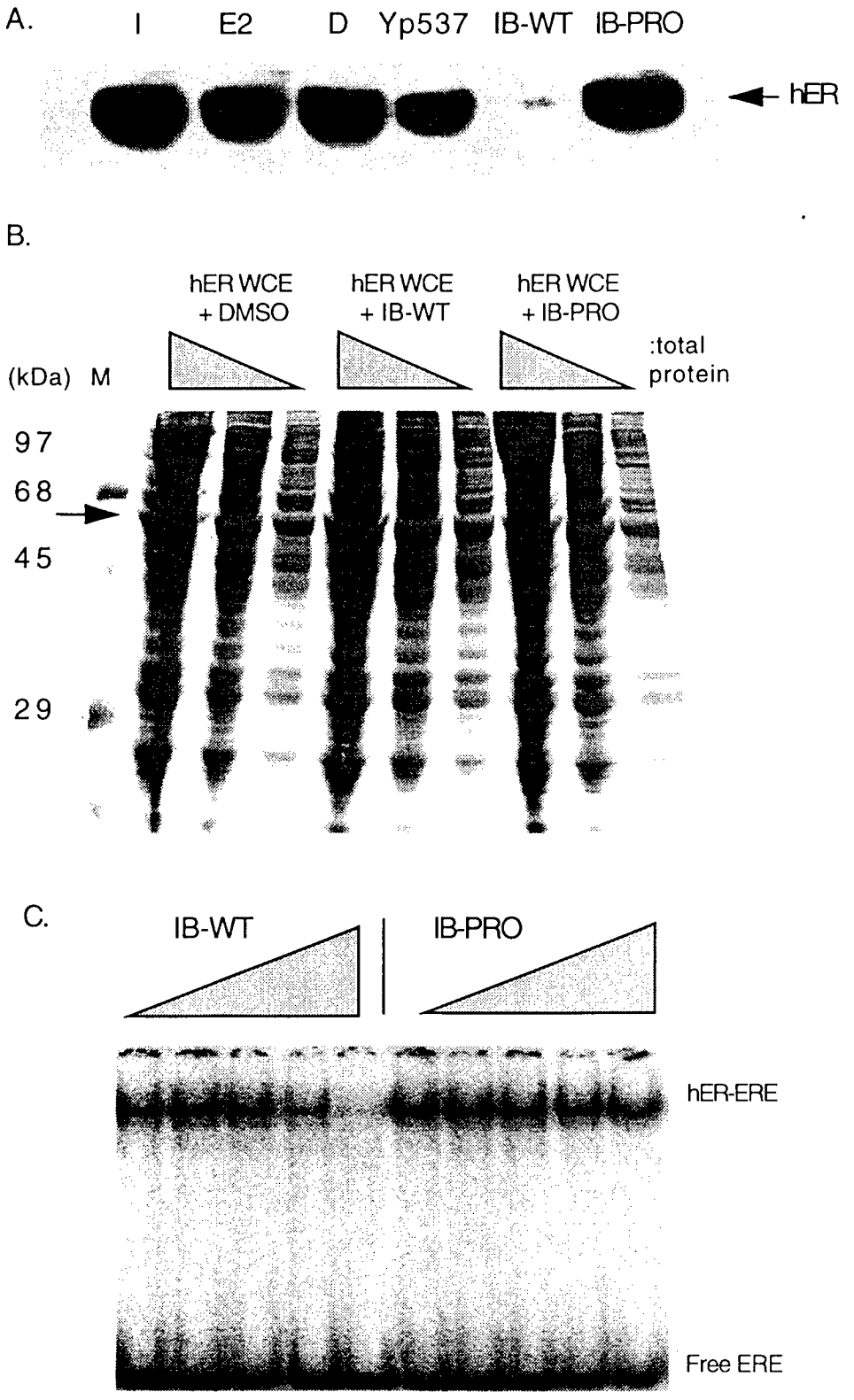
20
30
40
TFE (%)



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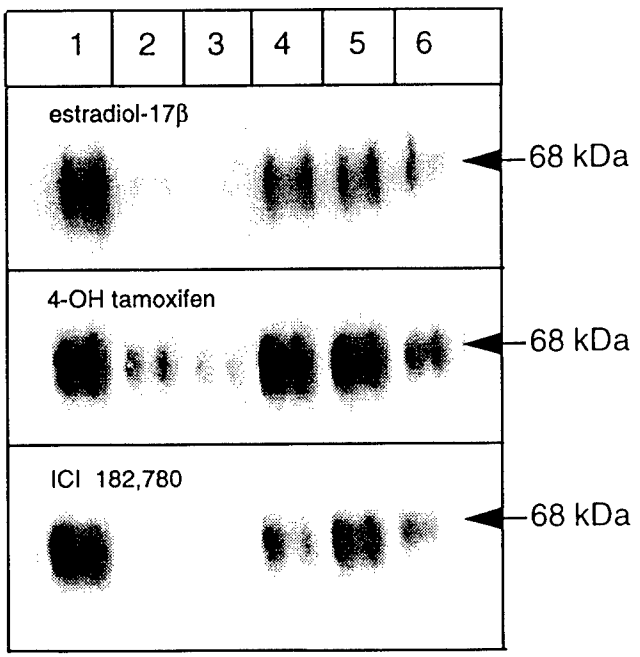
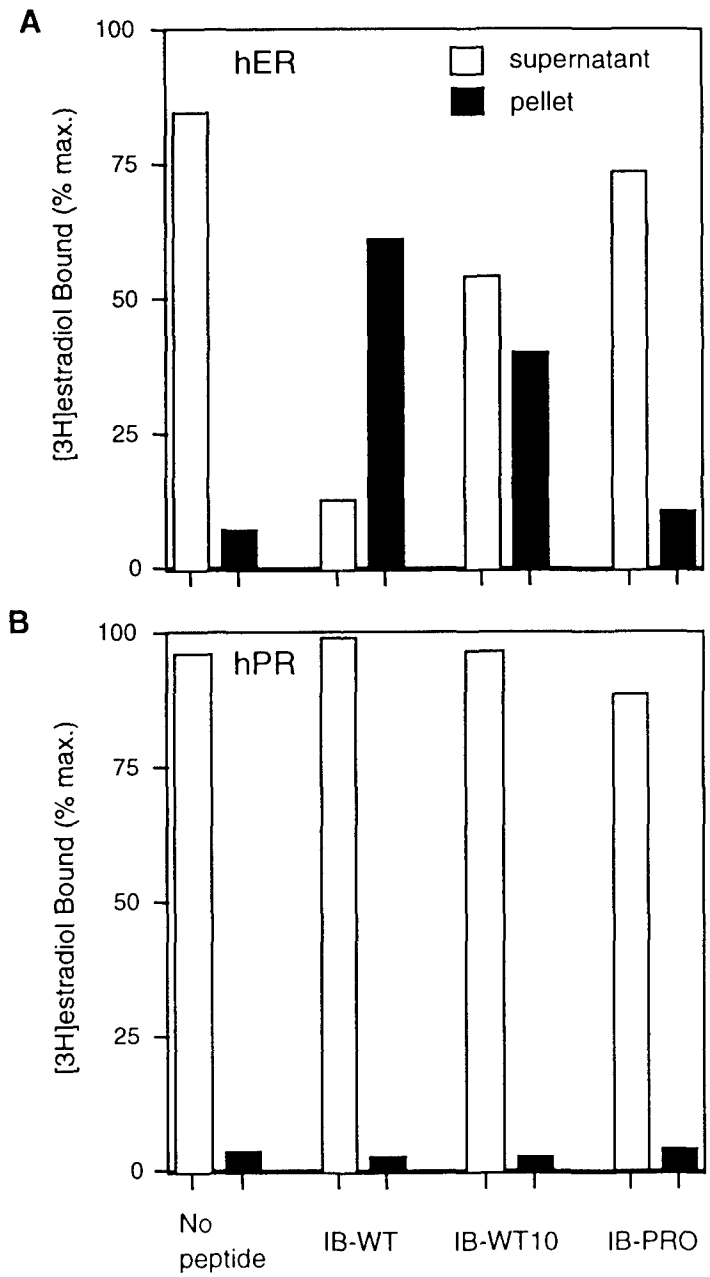
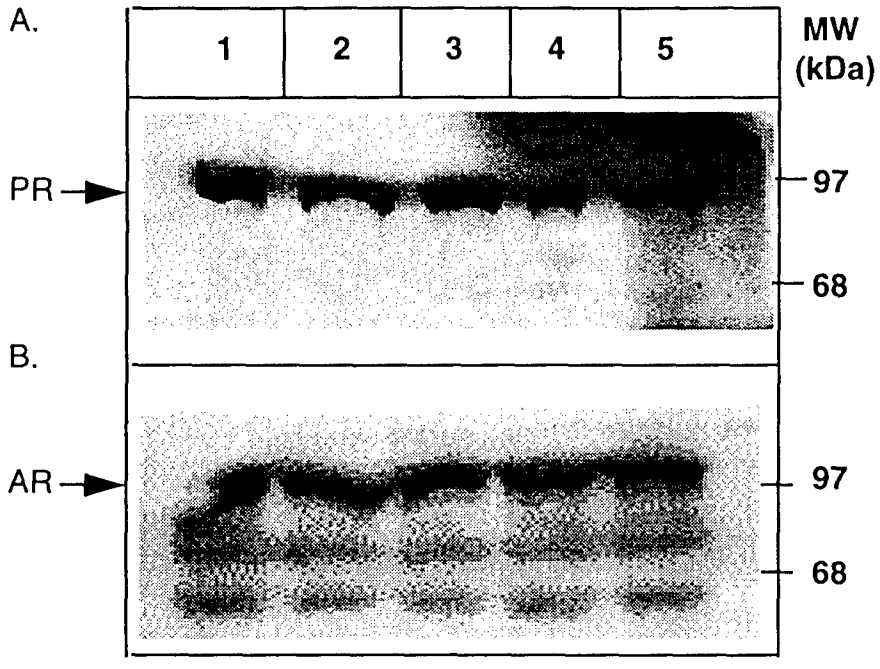


Yudt and Koide Figure 4.



Yudt and Koide, Figure 5.



Yudt and Koide, Figure 6.

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Supplemental Material

Ph.D. Thesis Exerts

The Role of Tyrosine 537 in Estrogen Receptor Function and the Development of Rationally Designed Peptide Antiestrogens

by

Matthew R. Yudt

Submitted in Partial Fulfillment
of the
Requirements for the Degree
Doctor of Philosophy

Supervised by
Professor Angelo C. Notides
and
Professor Shohei Koide

Department of Biochemistry and Biophysics
School of Medicine and Dentistry

University of Rochester
Rochester, New York
1998

Curriculum Vitae

The author was born in Allentown, PA on July 30, 1968. He attended the Pennsylvania State University from 1986 to 1990, and graduated with a Bachelor of Science degree in Chemistry. In September of 1993, he entered the Doctoral Program in Biophysics at the University of Rochester. He began his research in the laboratory of Angelo Notides in 1994 and received the Master of Science degree in 1996. Following the untimely death of Dr. Notides, he continued his research project under the guidance of Dr. Shohei Koide. In 1996 he was awarded a three-year pre-doctoral fellowship from the U.S. Army Medical Research and Materiel Command Breast Cancer Research Program. In 1998 he received the William F. Neuman Award in Biophysics. Upon completion of his Ph.D. in 1999, he will assume a postdoctoral position at the National Institute of Environmental Health Sciences studying the glucocorticoid receptor and apoptosis in the laboratory of Dr. John A. Cidlowski.

Abstract

An analysis of human estrogen receptor (hER) tyrosine 537 was carried out to elucidate its role in receptor function. Phosphorylation of this residue has been implicated in receptor dimerization, DNA- and hormone- binding, and transcriptional activation. A careful biochemical analysis of a tyrosine 537 to phenylalanine mutation revealed no significant changes in DNA or hormone binding affinities compared to wild type receptor. However, the Y537F hER mutation resulted in a decreased receptor stability, as measured by a loss of hormone and DNA binding over time relative to wild type receptor. I have concluded that phosphorylation of Y537 is not essential for hER function but Y537 is nevertheless a critical residue intricately involved with the conformation and ability of the hER to activate transcription.

A phosphotyrosine peptide derived from the hER sequence surrounding Y537 and containing part of helix 12 in the ligand binding domain is capable of blocking specific hER-DNA binding *in vitro*. The mechanism of this inhibition was studied to determine the potential of the peptide as a novel antiestrogen. The phosphopeptide inhibition of DNA binding requires the phosphotyrosine and the amino acids carboxy terminal to it. Analysis of hER deletion fragments suggests the ligand binding domain is required for phosphopeptide inhibition. However, the phosphopeptide does not bind to or compete with the ligand binding site.

The structural information of the ligand binding domain was exploited and a 'dimer-contact' oligopeptide was derived from the helical region of the wild type protein involved in receptor dimerization. This peptide, or I-box peptide, is capable of specifically precipitating the hER from cell extracts. Precipitation activity is correlated

with the helical nature of the peptide: a peptide containing a secondary structure-disrupting proline residue has no effect on hER function.

This study demonstrates the potential of small molecule inhibitors of hER to function as a novel class of antiestrogens which act independently from the hormone binding site. These interesting data suggest it is possible to rationally design peptides capable of blocking specific protein-protein interactions which may provide potential avenues for drug design and discovery.

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The Steroid Receptor Superfamily

The Role of Tyrosine 537 in Estrogen Receptor Function and the Development of Rationally Designed Peptide Antiestrogens

Matthew R. Yudt, Daria Vorojeikina, Angelo C. Notides, and Shohei Koide

Department of Biochemistry and Biophysics, School of Medicine and Dentistry; University of Rochester, Rochester, New York

An analysis of human estrogen receptor (hER) tyrosine 537 was carried out to elucidate its role in receptor function. Phosphorylation of this residue has been implicated in receptor dimerization, DNA- and hormone- binding, and transcriptional activation. A careful biochemical analysis of a tyrosine (Y) 537 to phenylalanine (F) mutation revealed no significant changes in DNA or hormone binding affinities compared to wild type receptor. Furthermore, in a yeast transactivation assay, the Y537F hER resulted in only a 25% loss of activity, relative to wild type receptor. Further analyses revealed that the Y537F hER mutation resulted in a decreased receptor stability, as measured by a time-dependent loss of hormone and DNA binding capacity. We have concluded that phosphorylation of Y537 is not essential for hER function but Y537 is nevertheless a critical residue intricately involved with the conformation and ability of the hER to activate transcription.

Relevant to these results is a previous report describing a phosphotyrosine peptide which is capable of blocking specific hER-DNA binding *in vitro*. This peptide is derived from the hER sequence surrounding Y537, and contains part of helix 12 in the ligand binding domain. The mechanism of hER inhibition was studied to determine the potential of the peptide as a novel antiestrogen. The phosphopeptide inhibition of DNA binding requires the phosphotyrosine and the amino acids carboxy terminal to it. Analysis of hER deletion fragments indicates the ligand binding domain is required for phosphopeptide inhibition. However, the phosphopeptide does not bind to or compete with the ligand binding site.

Structural information of the hER ligand binding domain was exploited and a 'dimer-contact' oligopeptide was derived from the helical region, of the wild type protein, involved in receptor dimerization. This peptide, or 'I-box peptide', is capable of specifically precipitating the hER from cell extracts. Precipitation activity is correlated with the helical nature of the peptide: a peptide containing a secondary structure-disrupting proline residue has no effect on hER function.

This study demonstrates the potential of small molecule inhibitors of hER to function as a novel class of antiestrogens which act independently from the hormone binding site. These data suggest it is possible to rationally design peptides capable of blocking specific protein-protein interactions which may provide potential avenues for drug design and discovery.

Funded by U.S. Army MRMC Breast Cancer Research Program grant DAMD17-96-1-6219 and NIH grant HD06707.

Abstract 2

A RATIONALLY DESIGNED PEPTIDE ANTIESTROGEN

Matthew R. Yudt* and Shohei Koide
Department of Biochemistry and Biophysics, University of Rochester

The human estrogen receptor (hER) is a therapeutic target and a clinical marker for estrogen responsive breast tumors. Recent advances in the development of hER antagonists, or antiestrogens, have raised optimism for the treatment and prevention of breast cancer and have contributed to our understanding of the structural and functional regulation of the estrogenic response. The majority of antiestrogens function by competing with the natural ligand, 17-β-estradiol, for binding to the hER.

We have exploited the structural information of the hER protein in developing a novel antiestrogen. Considering the functional hER is a homodimer, we have designed a 'dimer-contact' oligopeptide based on the helical region of the full length protein which is involved in dimerization. Based on circular dichroism spectroscopy, this peptide contains secondary structure similar to an alpha helix. The introduction of a proline residue in the center of this peptide sequence disrupts its secondary structure. The wild type peptide, or I-box peptide, appears to block hER dimerization and prevents the binding of the hER to its DNA response element in gel shift assays. Additionally, this peptide competes with the hormone estradiol, but through an alternate kinetic mechanism than observed with known hER antagonists. The proline-containing control peptide is completely void of all inhibitory properties associated with wild type I-box peptide sequence. The specificity of this peptide for the hER and the development of cellular models for studying the antiestrogen properties are being investigated. Our data suggests that rationally designed molecules capable of blocking protein quaternary structure may be potential avenues for drug design and discovery.

Funded by grants from the U.S. Army MRMC.

NEW ANTIESTROGENS. PHOSPHOPEPTIDES WHICH INHIBIT ESTROGEN RECEPTOR FUNCTION BY DISRUPTING DIMERIZATION.

Matthew R. Yudt, Enrique Castano, and Angelo C. Notides

The Department of Biochemistry and Biophysics, University of Rochester Medical Center, Rochester, NY, 14642

The human estrogen receptor (hER) is the protein which mediates the growth promoting effects of the sex steroid hormone estrogen. Nearly half of all breast cancers are estrogen receptor positive, meaning they express significantly more hER than estrogen negative breast cancers. Most of these cancers respond, at least initially, to antiestrogen therapy. Antagonists, such as tamoxifen, compete with the natural hormones for binding to the hER and inactivate the receptor, thereby inhibiting or diminishing tumor growth. However, in a rather unknown process, these cancers often develop antiestrogen resistance, or even worse, develop characteristics in which the antiestrogens become growth-promoting estrogens. The goal in our laboratory is to investigate the molecular mechanisms which regulate hER function with the intent to exploit these processes and control the function of the hER in the treatment or prevention or estrogen-positive breast cancer.

We have discovered a novel approach to inhibit the function of the hER. The DNA binding and transcriptionally active hER is a homodimer. Compounds which can disrupt the dimerization of the hER will inhibit these functions, thereby acting as pure antiestrogens. We have found that certain phosphotyrosine containing peptides are capable of specifically blocking hER dimerization, DNA binding and transcriptional activity.

Our results are based on several *in-vitro* biochemical techniques. First, specific phosphotyrosine peptides are shown to block the DNA binding function of the hER. An 11 amino acid phosphotyrosyl peptide, whose sequence is derived of residues 532 to 542 surrounding phosphotyrosine 537 of the hER, can specifically block estrogen response element (ERE) binding in gel mobility shift assays. The specificity of this interaction was further investigated using seven amino acid phosphotyrosyl peptides where it was found that the residues C-terminal to the Y537 (i.e., 536 to 542) are necessary and sufficient for blocking hER dimerization. Secondly, these peptides used in a cell-free system were found to inhibit the transcriptional activation of the hER, the quintessential function of hER. It is further shown, using molecular sizing chromatography, that these peptides work through similar mechanisms and specifically interact with the hER to block dimerization.

In conclusion, we demonstrate a novel approach to block the function of the hER using phosphotyrosine peptides to disrupt hER dimerization. These peptides represent a potentially new class of antiestrogens which appear to function with a completely different mechanism than the classical steroidal antiestrogens.

Keywords: Human Estrogen Receptor, Phosphotyrosine, Dimerization, Peptide, Antiestrogen

This work was supported by the U.S. Army Medical Research and Materiel Command under DAMD-17-96-1-6219.

Abstract 4

DISRUPTION OF ESTROGEN RECEPTOR DIMERIZATION AND DNA-BINDING BY A 12-MER PHOSPHOTYROSYL PEPTIDE REQUIRES THE C- BUT NOT N-TERMINAL AMINO ACIDS.

M.R. Yudt, E. Castano, and A.C. Notides, Department of Biophysics, University of Rochester, Rochester, NY, 14642.

We have previously shown that phosphorylation of the human estrogen receptor (hER) on tyrosine 537 is a prerequisite for dimerization, DNA-binding, and activation of transcription. A 12 amino acid phosphotyrosyl peptide, whose sequence corresponds to residues 532 to 542 surrounding phosphotyrosine 537 of the hER, can specifically block hER dimerization and DNA-binding. These data suggest that the mechanism of hER dimerization involves reciprocal interaction of hER monomers through the phosphotyrosine of one monomer and a phosphotyrosine binding domain of the other. We have further investigated the specificity of phosphotyrosyl peptide disruption of hER dimerization and DNA-binding. A seven amino acid phosphotyrosyl peptide including residues N-terminal to Y537 (i.e., 532 to 538) failed to block hER dimerization. In contrast, a phosphotyrosyl peptide including the residues C-terminal to the Y537 (i.e., 536 to 542) are necessary and sufficient for blocking hER dimerization as seen by gel mobility shift assays, far western blot analysis and *in vitro* transcription assays. Furthermore, changing the first or the third amino acid C-terminal to the phosphotyrosine (i.e., aspartate 538 to asparagine, or leucine 540 to alanine) results in peptides unable to block hER dimerization, indicating that aspartic acid 538 and leucine 540 are additional determinants to the phosphotyrosine moiety for the phosphotyrosine-binding domain. These results indicate that the mechanism for hER dimerization is a phosphotyrosine/phosphotyrosine-binding domain coupling of monomers and that the specificity of the phosphotyrosine binding domain for C-terminal amino acids is similar to the classical SH2 domain of $p60^{c\text{-}src}$.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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Rec'd 10/27/2000

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